12-2013

Characterization of the Hydrogen Peroxide Stress Responses of Bifidobacterium longum and Bifidobacterium animalis subsp. Lactis

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CHARACTERIZATION OF THE HYDROGEN PEROXIDE STRESS RESPONSES OF

BIFIDOBACTERIUM LONGUM AND *BIFIDOBACTERIUM ANIMALIS* SSP. *LACTIS*

by

Taylor Oberg

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Nutrition and Food Sciences

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UTAH STATE UNIVERSITY
Logan, Utah

2013

ABSTRACT

Characterization of the Hydrogen Peroxide Stress Responses of *Bifidobacterium longum* and *Bifidobacterium animalis* subsp. *lactis*

by

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Utah State University 2013

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Consumer interest in probiotic bifidobacteria is increasing, but industry efforts to secure high cell viability are undermined by the sensitivity of these anaerobes to oxidative stress during food production or storage. In this study, we examined the physiological and transcriptional stress responses of three strains of *Bifidobacterium longum* and three strains of *Bifidobacterium animalis* subsp. *lactis* to hydrogen peroxide (H_2O_2). Intrinsic and inducible H_2O_2 resistance was determined for each strain, and results showed *B. longum* subsp. *infantis* ATCC 15697 had the highest level of intrinsic H_2O_2 resistance. Inducible H_2O_2 resistance was detected in four strains, *B. longum* strains NCC2705 and D2957, *B. animalis* subsp. *lactis* strains RH-1 and BL-04.

We then examined the transcriptional responses of *B. animalis* subsp. *lactis* strains, BL-04 and DSM10140, and *B. longum* strains NCC2705 and D2957 to H_2O_2

exposure. Transcriptional analysis was performed on cells exposed to a sub-lethal H_2O_2 concentration for 5, 20, or 60 min compared to an untreated control. *B. animalis* subsp. *lactis* BL-04 showed differential expression (DE) in 158 genes after 5 min, and 30 genes after 60 min. Surprisingly, no significant DE genes were detected in *B. animalis* subsp. *lactis* DSM10140 at either time. Although the strains are virtually identical genetically, genomic data suggested differences in H_2O_2 stress resistance might be related to the function of long chain fatty acid-coA ligase. To address this hypothesis, membrane fatty acids were isolated and analyzed by GC-MS. Results confirmed the two strains had significantly different lipid profiles, which could affect membrane fluidity and, potentially, transduction of stress signals.

Data for the *B. longum* strains showed NCC2705 had 316 DE genes after the 5-min treatment and 131 DE genes after the 20-min treatment. In contrast, the D2957 strain had only 24 and 116 DE genes after the 5- and 20-min treatments, respectively.

These data indicate that intrinsic and inducible resistance to hydrogen peroxide is strain specific, and suggest that for some strains, sublethal H_2O_2 treatments and cell membrane modifications might help increase cell resistance to oxidative damage during production and storage of probiotic-containing foods.

(192 pages)

PUBLIC ABSTRACT

Characterization of the Hydrogen Peroxide Stress Responses of *Bifidobacterium longum* and *Bifidobacterium animalis* subsp. *lactis*

Taylor S. Oberg

Probiotics are living organisms which exert a beneficial health effect when consumed in sufficient numbers. Consumer interest in probiotics has increased dramatically in recent years prompting an increase in production and development of functional foods. One major problem is the decreased viability of probiotic bacteria during functional food production and storage and subsequent digestion due to environmental stresses. The most common probiotic strains belong to the genus *Lactobacillus* or *Bifidobacterium*. Due to the anaerobic nature of these bacteria, they lack the required defense mechanisms for oxidative stress inherent in aerobic microorganisms. This study examined the oxidative stress responses of six strains of *Bifidobacterium,* which are commonly used as probiotics in functional foods

The first phase of the study investigated the innate and inducible hydrogen peroxide (H_2O_2) stress response of *Bifidobacterium longum* strains NCC2705 and D2957, *Bifidobacterium longum* ssp. *infantis* ATCC 15697, and *Bifidobacterium animalis* ssp. *lactis* strains BL-04, DSM10140 and RH-1. Strains were screened for survival at increasing concentrations of H_2O_2 and lethal and sublethal concentrations were determined for each. In the second phase, *B. animalis* ssp. *lactis* strains BL-04 and DSM10140 and *B. longum* strains NCC2705 and D2957 were

treated with a sublethal H_2O_2 concentration and RNA samples were collected for transcriptome analysis after 5 min and either 20 or 60 min. Statistical analysis was performed to identify genes that increased or decreased in expression during H_2O_2 treatment compared to control cells.

Results showed that survival was species and strain dependent and that strains which naturally survived higher H_2O_2 concentrations had a larger number of differentially expressed genes early on during H_2O_2 exposure. Some of the protective genetic systems that were activated during H_2O_2 stress are mechanisms which perform basic cellular functions under normal conditions such as deoxuynucleotide synthesis. Under stress conditions, these systems can be used to detoxify oxidative free radicals. Also a number of genes involved in sugar transport and energy production for the cell showed increased expression, which reveals the increased energy needs of the cells during oxidative stress.

During testing, it was found that two *B. animalis* ssp. *lactis* strains, BL-04 and DSM10140, had differing levels of survival and gene expression during H_2O_2 exposure despite having almost identical genome sequences. It was determined that one possible cause of the differences was a genetic deletion in a gene that allows the cell to incorporate extracellular fatty acids into the cell membrane instead of synthesizing them.

Results from this project have increased the understanding of oxidative stress responses in bifidobacteria and highlighted possible methods to increase bacterial survival during food manufacture, storage, and human digestion.

DEDICATION

I woud like to dedicate my dissertation to my wife, who happily stood by me through all of this, and to my daughters; there is no boundry to what you can achieve.

ACKNOWLEDGMENTS

I would first like to thank my advisor and mentor, Dr. Jeff Broabent, for his encouragment, trust, guidance, and support during my time as a graduate student. It has been an honor to receive training and direction from one of the great instructers and researchers in the latic acid bacteria field. I would also like to give recognition to the Nutrition, Dietetics and Food Science faculty for all of the instruction and impromptu advice and guidance. I extend thanks to the collaborators of this project, mainly Dr. James Steele at the University of Wisconsin, and to the members of my graduate committee for their time commitment in promoting my success as a student.

I would also like to thank my parents for teaching me the value of education through their example and for their constant support of my decisions and endevors no matter how crazy. Finally I would like to thank my wife who has supported and lifted me up through all of this; I could never have done this without you.

This project was supported by National Research Initiative Grant no. 2006-35503-17194 from the USDA Cooperative State Research, Education, and Extension Service *Improving Food Quality and Value Program*, and by the Utah Agricultural Experiment Station.

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CHAPTER 1

INTRODUCTION AND OBJECTIVES

Food or food ingredients with "bioactive" properties have increased in popularity among consumers over the last few years. These functional foods are defined by their ability to impact human health in a manner that is not based only on their nutritional value. As such, probiotic bacteria represent one of the most promising categories of bioactive food ingredients (1). The term "probiotic" commonly refers to "living organisms which, when ingested at sufficient numbers, exert a beneficial effect on the host organism beyond inherent general nutrition" (2).

Currently, species of *Lactobacillus* and *Bifidobacterium* are the most widely used probiotic bacteria added to commercial products (3). Though they are relatively minor components of the normal gastrointestinal microbiota in human adults, bifidobacteria are thought to promote or provide several health related functions, including host resistance to infectious microbes, anti-carcinogenic activities, and improved nutritional efficiency (4). Although many species of bifidobacteria are used as probiotics, the two most important commercial species are *Bifidobacterium animalis* subsp. *lactis* and *Bifidobacterium longum*.

Researchers have suggested that probiotic bacteria should generally satisfy several criteria, including the ability to maintain high viability during the technological processes used to prepare and deliver the bacterium, and the ability to survive passage through the upper GI tract for adherence and colonization of the large intestine (5, 6, 7). Although no conclusive data is available on a minimal

effective dose of probiotics in humans, results from several clinical trials suggest a direct dose-effect correlation (8, 9, 10). As a result, the current WHO definition of probiotics emphasizes the need for administration of the probiotics in "adequate amounts" (11). Thus, successful application of probiotic bifidobacteria in foods is not only dependent upon scientific research that demonstrates their efficacy in providing human health benefits, but also on the development of technologies to ensure their survival in high numbers during food processing and maintaining those high numbers during storage.

Industrially, bifidobacteria are usually preserved or distributed in liquid, spray-dried, frozen, or lyophilized forms (12, 13). Most of the currently used culture preservation and production methods subject cells to stress conditions, such as freezing, drying, nutrient starvation, and concentration stress, that serve to diminish cell viability (13). Difficulties also arise with probiotic bifidobacteria applications due to the fact that cells are typically exposed to stress conditions during the manufacturing process of most food-based delivery systems (14). At present, yogurt or fermented milks are the most common vehicle foods for delivery of probiotic bifidobacteria, but regardless of the vehicle food, efforts to secure and maintain high numbers of viable bifidobacteria in the product are commonly impeded by the intrinsic properties of the food such as dehydration (low A_w), high or low temperature, low pH, and elevated oxygen or NaCl levels, all of which are deleterious to bifidobacteria (14-17). To address this problem, processors may

employ very large inocula or add specific growth promoters or protectants (14, 18, 19).

Understanding the requirements for preservation methods that promote high cell viability and retained metabolic activity among probiotic bacteria can present a formidable challenge. Fortunately, some species and strains are intrinsically more resistant to environmental stresses than are others, which indicate that this limitation can probably be overcome through a more fundamental understanding of bifidobacteria physiology and their molecular mechanisms for cell protection during environmental stress. For this reason, there has been a growing worldwide interest in bifidobacteria stress responses and their potential application to promote cell survival during processing, distribution, and consumption of probiotic foods (14, 17, 20). Outcomes from this research will provide a more complete and detailed understanding of how these *Bifidobacterium* respond to H_2O_2 stress conditions encountered during food production or storage, and will identify potential strategies to enhance long-term cell survival in bioactive foods. Research described in this dissertation addressed the following objectives:

1. Characterize physiological stress responses among *B. animalis* subsp. *lactis* and *B. longum* strains to oxidative stress caused by exposure to H_2O_2.
    a. Determine the lethal and sublethal H_2O_2 concentrations for each strain.
    b. Define the fatty-acid composition change in the bacterial cell wall that occurs during the oxidative stress response.

2. Characterize the inducible stress response of *B. lactis* and *B. longum* to sublethal oxidative stress levels caused by exposure to H_2O_2.
3. Use DNA microarray technology to identify and define similarities and differences in the transcriptional responses of specific strains from each species that display an intrinsic and inducible stress response to H_2O_2.

Strains selected for use in this study represent cultures that are industrially important and had available genome sequence data to support DNA microarray studies. Previous studies have shown a varying degree of resistance in bifidobacteria and lactic acid bacteria at a wide range of H_2O_2 concentrations tested in differing buffers (21-26). To accomplish Objective 1, strains were treated with a range of H_2O_2 concentrations in a growth media instead of a buffer, allowing cells the opportunity for active metabolism during exposure. The concentration of H_2O_2 in the medium was also measured during the exposure period to monitor possible degradation. This data were then used to define a lethal and sublethal H_2O_2 concentration for each strain.

The cell membrane composition plays an important role as the first line of defense against acid stress. A modified version of the MIDI laboratories protocol for cell membrane fatty acid analysis (27) was used to determine if the cell membrane fatty acid profile changes during H_2O_2 exposure in bifidobacteria.

In many bacteria, including species of bifidobacteria, it has also been shown that cell survival may be dramatically improved by deliberate induction of an adaptive or inducible stress response (28-37). To accomplish objective 2, strains

were exposed to previously defined sublethal H_2O_2 concentrations for set intervals, and these pretreated cells were then subjected to lethal concentrations of H_2O_2. An inducible stress response was defined as an increase in survival at lethal H_2O_2 levels after pre-exposure to sublethal H_2O_2 concentrations compared to unexposed cells.

DNA microarray technology has vastly improved the ability of researchers to determine the influence of gene expression in microbial adaptation to different environmental conditions. To accomplish objective 3, DNA microarrays containing all of the unique open reading frames for both *B. longum* NCC2705 and *B. animalis* subsp. *lactis* strains DSM10140 and BL-04 were designed by our lab group and obtained from Affymetrix (Affymetrix, Santa Clara, CA). RNA was isolated from cells exposed to sublethal H_2O_2 concentrations and used to generate cDNA for hybridization to the custom DNA microarrays. Raw data from the hybridization was analyzed using the Bioconductor packages in the open source statistical platform R (www.r-project.org). Statistically differentially expressed genes were then grouped according to cellular function for comparison between strains.

Although there has been much research performed on the stress responses of *Bifidobacterium* species, there is a gap in the understanding of oxidative stress responses of these bacteria. Results from this study have helped define the intrinsic resistance and inducible stress responses of bifidobacteria to H_2O_2 through physiological studies and transcriptomics. This understanding can be used as a basis for creating methods to increase probiotic bifidobacteria survival in food production and storage.

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CHAPTER 2

LITERATURE REVIEW

Functional foods

Functional foods are foods or food ingredients with "bioactive" properties which are defined by their ability to impact human health in a manner that is not based only on their nutritional value. Consumer interest in such functional food products has increased dramatically over the last few years. As such, probiotic bacteria represent one of the most promising categories of bioactive food ingredients (1). The term "probiotic" commonly refers to "living organisms which, when ingested at sufficient numbers, exert a beneficial effect on the host organism beyond inherent general nutrition" (2). Currently, species of *Lactobacillus* and *Bifidobacterium* are the most widely used probiotic bacteria added to commercial products (3).

Bifidobacteria

The *Actinobacteria* phylum is one of the largest taxonomic units among the *Eubacteria* and is comprised primarily of Gram positive bacteria with a high guanine + cytosine (G+C) content (>51%). This phylum includes the *Bifidobacteriales* order, containing only one family, *Bifidobacteriaceae,* which consists of 8 genera with *Bifidobacterium* as the only genus with multiple species (4, 5).

Bifidobacterium species are Gram-positive, non-acid-fast, non-spore forming, non-motile, catalase negative rods of irregular shape, and they are anaerobic

chemoorganotrophs that metabolize a variety of carbohydrates through fermentation to produce organic acids but not gas (6). Bifidobacteria species have been isolated from multiple sources including food, sewage, insects, the human oral cavity, and the guts of both humans and animals (7). Some species have been isolated from both human and animal gut, showing the ability to grow in different hosts, whereas other species have only been isolated from the gut of certain animal species (eg., rabbits, cows and chickens) demonstrating a highly specialized adaptation to specific ecological environments (8, 9, 10). This highly evolved adaptation to growth in the gut is illustrated by the fact that strains can ferment complex carbohydrates for growth that are not digestible by the host (11-14). Genetic analysis has revealed numerous oligosaccharide transporters and glycosyl hydrolases that can account for up to 10% of the genomic content of some strains (12, 15, 16).

This specialization in ecological adaptation is illustrated by comparison of *B. longum* subsp. *infantis* ATCC 15697, a strain commonly isolated in human infants, and *B. animalis* subsp. *lactis* strain, which are commonly isolated from human adults. The genome of *B. longum* subsp. *infantis* ATCC 15697 contains genes encoding proteins involved in the breakdown and utilization of human milk oligosaccharides in breast milk, which are not found in the genomes of *B. animalis* subsp. *lactis* strains (13). Instead, *B. animalis* subsp. *lactis* strains contain a large number of genes involved in breakdown and metabolism of complex carbohydrates found in an adult-type diet containing plant derived fibers (12, 17).

Energy production and fermentation

Although a majority of the *Bifidobacterium* species can utalize complex carbohydrates, most strains will favor simple sugars over more complex sugars if they are available. *Bifidobacterium* species lack genes involved in the electron transport chain for oxidative phosphorylation and degrade hexose sugars by fermentation exclusively through the fructose-6-phosphate phosphoketolase (F6PK) pathway commonly known as the "bifid shunt" (6, 18). The key enzyme in this pathway, F6PK, cleaves hexose phosphate to erythrose-4-phosphate then acetyl phosphate, which is converted to acetate by substrate level phosphorylation. Pentose phosphates are also generated from tetrose and hexose phosphates using transaldolase and transketolase, which are then degraded to acetyl phosphate and glyceraldehyde-3-phosphate, which is then oxidized to pyruvate via the Emden-Myerhoff pathway. Pyruvate is then reduced to lactate to regenerate NAD^+, giving a theoretical yield of 3 mol acetate and 2 mol of lactate for every 2 mol of glucose consumed (19, 20). This pathway functions in bifidobacteria due to the fact that they lack several genes involved in glycolysis, including the key regulatory enzyme phosphofructokinase.

Probiotic health benefits

Though they are relatively minor components of the normal gastrointestinal (GI) microbiota in human adults, bifidobacteria are thought to promote or provide several health related functions (21, 22). Moreover, certain species of bifidobacteria are major components of the GI microbiota of healthy, breast-fed infants, and recent

work suggests the composition of GI microbiota in infants and children may influence the development of diarrheal, inflammatory, and allergic diseases (23).

The relationship between bifidobacteria and human health has intrigued physicians and microbiologists for over 100 years (24), and stimulated consumer interest in bioactive foods that contain bifidobacteria as seen recently with new products on the market (25, 26). Examples of probiotic effects documented with the consumption of bifidobacteria include a decrease in severity of the side effects associated with antibiotics use, a reduced incidence of infection in patients receiving irradiation therapy, a decrease in the duration of diarrhea due to various etiologies, improved lactose digestion, a reduced frequency of allergic reactions, and normalization of blood lipid composition (3, 27, 28, 29, 30). Recent clinical trials have shown that bifidobacteria can alleviate lactose malabsorption symptoms, increase bowel transit time and stool frequency in children and adults suffering constipation, be used as an effective treatment for ulcerative colitis, alleviate symptoms of irritable bowel syndrome, reduce the symptoms of stress induced GI disorders and stimulate the immune system of patients with HIV infection to increase the overall quality of life (31-39).

Although many species of bifidobacteria are used as probiotics, the two most important commercial species are *Bifidobacterium animalis* ssp. *lactis* and *Bifidobacterium longum*. The ability of these and other species of bifidobacteria, to exert probiotic activities is thought to result from the combination of direct (e.g., antagonism of pathogens via the production of antimicrobials or competition for

attachment sites) and indirect (e.g., immuno-stimulation) mechanisms (3, 22, 40). Researchers have suggested that to serve as an effective probiotic agent, these bacteria generally should satisfy several criteria including: (i) be able to exert a beneficial effect towards the host; (ii) be non-pathogenic and non-toxic; (iii) be able to survive passage through the upper GI tract; (iv) be able to adhere to and colonize the large intestine; (v) be derived from the same species as the intended host; (vi) show antimicrobial and /or immunomodulatory activity; and (vii) be able to maintain a high viability during the technology processes used to prepare and deliver the bacterium (41-44). Although no conclusive data is available on a minimal effective dose of probiotics in humans, results from several clinical trials suggest a direct dose-effect correlation (45, 46, 47). As a result, the current WHO definition of probiotics emphasizes the need for administration of the probiotics in "adequate amounts" (48). Thus, successful application of probiotic bifidobacteria in foods is not only dependent upon scientific research that demonstrates their efficacy in providing human health benefits, but also on the development of technologies to ensure their survival in high numbers during food processing and maintaining those high numbers during storage. While research efforts are currently underway to demonstrate the efficacy of bifidobacteria in promoting or providing various health benefits, knowledge of methods to enhance the survival of bifidobacteria in food-based delivery systems remains fragmentary.

Probiotic food production

Industrially, bifidobacteria are usually preserved or distributed in liquid, spray-dried, frozen, or lyophilized forms (49, 50). Unfortunately, understanding the requirements for preservation methods that promote high cell viability and retained metabolic activity among probiotic bacteria can present a formidable challenge. This is because most culture preservation and production methods subject cells to stress conditions, such as freezing, drying, nutrient starvation, and concentration stress, that serve to diminish cell viability (50). As a result, problems in large-scale cultivation and preservation of bifidobacteria continue to limit the commercial application of these cultures (51).

Difficulties associated with probiotic strain production are further exacerbated by the fact that exposure to cellular stress conditions are also an integral feature of the manufacturing process for most food-based delivery systems for bifidobacteria (51). At present, yogurt or fermented milks are the most common vehicle foods for delivery of probiotic bifidobacteria, but cheese, ice cream, infant formula, fruit juice, and other foods are also used to a lesser extent (51, 52). Regardless of the vehicle food, efforts to secure and maintain high numbers of viable bifidobacteria in the product are commonly impeded by the intrinsic properties of the food such as dehydration (low A_w), high or low temperature, low pH, and elevated oxygen or NaCl levels, all of which are deleterious to bifidobacteria (52-55). To address this problem, processors may employ very large inocula or add specific growth promoters or protectants (51, 56, 57). To function as an effective probiotic,

strains must also be able to survive gastric stresses, primarily the low pH of the stomach and bile salt exposure in the small intestine (58, 59).

Micro-encapsulation is another promising technology to enhance survival of bifidobacteria in foods. This process surrounds cells with various matrices, such as calcium alginate, pectin, or whey proteins, to protect them from adverse environments in the food and during transit through the upper GI tract (25, 60). Though encapsulation can impart significant protection to some strains of bifidobacteria, wide differences in strain robustness continue to hamper industry efforts to secure high numbers of viable shelf-stable probiotic bacteria in food products (51, 60).

Oxidative stresses pose a major hurdle in the application of bifidobacteria in functional foods due to their anaerobic nature. Oxygen toxicity results from cell exposure to activated oxygen compounds such as superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radicals, which cause peptide breaks, oxidation of sulfhydryl groups in proteins, DNA damage and oxidation of membrane lipids (61). Although H_2O_2 is the most stable of these molecules, dissociation and interaction with cellular components can form organic peroxides, which can initiate a chain reaction of oxidation (62). One common source of H_2O_2 is from metabolic byproducts of the starter cultures used to manufacture yogurt, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus,* which have been shown to produce inhibitory concentrations of H_2O_2 when grown under conditions such as those encountered during bioactive food production and storage (63).

Acid stress responses of bifidobacteria

One of the primary problems that cells negotiate during stress is protein damage resulting in misfolded and non-functioning proteins. To overcome this problem, cells utilize molecular chaparones for proper protein folding and stabilization during stresses and, if the protein is damaged beyond repair, ATP dependent proteases hydrolyze proteins to prevent accumulation of non-functioning proteins in the cell (64, 65, 66). Genetic analysis of *B. animalis* subsp. *lactis* and *B. longum* strains reveal a small assortment of classical molecular chaparones and stress-associated proteases (67, 68).

The detrimental effects of acid stress on bifidobacteria are caused by elevated proton concentrations. At low pH, undissociated organic acids can passively diffuse across the membrane, and once internalized, dissociate and acidify the cytoplasm, causing metabolic failure and eventually cellular death. Overall, bifidobacteria survive poorly at low pH, with the exception of *B. animalis* subsp. *lactis* strains, which is why strains from this species are widely used in functional foods (69, 70). Studies have shown that in anaerobic bacteria like bifidobacteria, the main cellular defense against low pH is the F_0F_1-ATPase, which pumps protons across the cytoplasmic membrane at the expense of hydrolyzing ATP (71, 72, 73). This survival mechanism has been confirmed in *B. animalis* subsp. *lactis* and *B. longum* strains through proteomic and transcriptomic data (74, 75, 76). This same data has shown that in addition to the classical stress response chaperones (eg.,

DnaK, DnaJ, GroEL, GroES), acid stress also caused the increase of proteins involved in the glycolytic pathway (75). Induction of genes involved in metabolism shows that the cells are rerouting metabolites to different branches, including the P6PK pathway, to modify energy production and redox equivalents. This underscores the increased energy needs for the cells during stress exposure for proton expulsion and protein synthesis. Research has also shown that low pH increases the amount of enzymes involved in branched chain amino acid biosynthesis as well as glutamine synthetase (75). Branched chain amino acid synthesis is coupled to the conversion of glutamine to glutamate liberating an ammonia ion, which acts as a cellular buffer (77, 78).

Bile stress responses of bifidobacteria

Human bile is a mixture of electrolytes, bile salts, phospholipids, cholesterol, bilirubin and proteins, which is secreted into the duodenum during digestion to facilitate emulsification and absorption of lipid soluble nutrients. A certain percentage of bile salts escape entero-hepatic circulation and act as strong antimicrobials in the colon due to their strong detergent action, which damages cellular membranes and interrupts membrane functionality. Oxidative stress is a well-known function of bile salt exposure in cells and has been shown to cause DNA damage and generate protein conformation modifications and increase protein turnover (79, 80, 81). Because of this, bile salt exposure to bifidobacteria will trigger oxidative stress mechanisms in response to bile such as the thioredoxin reductase system, along with the classic stress response chaperones (81, 82).

The cellular membrane is the first line of defense against cellular stress and bacterial cells have developed methods for rapid modification of the fatty acid moieties and head groups in response to stress. Research has shown that adding Tween 80 to the growth media of some LAB strains consequently increases their survival at low pH (83), and that bile stress induces modification of the membrane fatty acid composition in *B. animalis* subsp. *lactis,* which is accomplished through incorporation of fatty acids with different chain lengths, saturation, or post synthetic modification of existing fatty acids (84, 85). In *B. animalis* subsp. *lactis,* bile salt exposure decreased the production of a long-chain fatty acyl CoA ligase, which is used for incorporation of exogenous fatty acids into the cellular membrane (86).

Oxidative stress responses of bifidobacteria

Oxidative stresses in bacteria are caused by reactive oxygen species such as the superoxide anion, H_2O_2, or hydroxyl radicals, which damage biomolecules through mechanisms such as; oxidation of sulfhydryl groups and peptide breaks in protein, fatty acid peroxidation and propagation, and DNA damage through depolymerization and depurination (87, 88, 89).

Most bacteria possess classical oxidative defense mechanisms to detoxify oxidative free radicals such as super oxide dismutase, catalase and NADH peroxidases (90, 91). Because of the anaerobic nature of bifidobacteria and their adaptation to the anaerobic environment of the gut, they lack these classical enzymes for oxygen radical detoxification. Genetic analyses of *B. animalis* subsp. *lactis* and *B. longum* strains reveal that they contain genes for flavin proteins,

although they lack a gene for flavin reductase, as well as genes for a thioredoxin reductase-thioredoxin system and a peroxiredoxin (67). Ferritin-like iron binding proteins have also been identified as a protective mechanism against oxidative free radicals by sequestering iron to prevent free hydroxal radical formation (92).

Molecular understanding of oxidative stress responses in bifidobacteria, particularly H_2O_2, is limited. Previous studies have investigated H_2O_2 resistance in bifidobacteria and LAB, though due to experimental design, an increased understanding of the mechanism was not provided. This was partially due to a wide range of H_2O_2 concentrations tested under static conditions in differing buffer solutions at very short or very long exposure times (93-97).

Inducible stress responses and crossprotection

Several studies have shown that survival of many bacteria, including species of bifidobacteria, under stress conditions may be dramatically improved by deliberate induction of an adaptive or inducible stress response (98-105). These bacterial stress responses are characterized by the transient induction of specific genes that encode for protective proteins (106). Although specific information on this topic in bifidobacteria is still quite limited, researchers have confirmed stress treatments are followed by the coordinated expression of gene products that include general and specific stress proteins (e.g. chaperones and ATP-dependent proteases) and corresponding regulatory proteins (82, 107, 108, 109, 110).

In the context of industrial or medical microbiology, one of the more significant facets of bacteria stress adaptation is the finding that stress responses

induced under one set of conditions commonly provide cells with significant cross protection against other hostile yet seemingly unrelated environments (111). Recent studies have shown that the breadth and degree of environmental resistance or cross protection induced by a particular stress treatment can differ significantly between species and even strains (52, 104, 105, 112, 113, 114, 115). Although the knowledge that sublethal stress treatments can promote cell robustness is already exploited in the manufacture and use of probiotic cultures (55), a more detailed understanding of environmental adaptation specifically targeting bifidobacteria should reveal new strategies to improve the industrial stability, performance, and utility of these probiotic bacteria.

Fortunately, some species and strains of bifidobacteria are intrinsically more resistant to environmental stresses than are others, which indicates that this limitation can probably be overcome through a more fundamental understanding of bifidobacteria physiology and their molecular mechanisms utilized during environmental stress response.

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CHAPTER 3[1]

INTRINSIC AND INDUCIBLE STRESS RESPONSES TO HYDROGEN PEROXIDE IN *BIFIDOBACTERIUM* SPECIES

ABSTRACT

Interest in, and use of, bifidobacteria as a probiotic delivered in functional foods has increased dramatically in recent years. Due to their anaerobic nature, oxidative stress can pose a major challenge to maintaining viability of bifidobacteria during functional food storage. To better understand the oxidative stress response in two industrially important bifidobacteria species, we examined the response of three strains of *Bifidobacterium longum* and three strains of *Bifidobacterium animalis* subsp. *lactis* to hydrogen peroxide (H_2O_2). Each strain was exposed to a range of H_2O_2 concentrations (0 mM to 10 mM) to evaluate and compare intrinsic resistance to H_2O_2. Next, strains were tested for the presence of an inducible oxidative stress response by exposure to a sublethal H_2O_2 concentration for 20 or 60 min followed by challenge at a lethal H_2O_2 concentration. Results showed *B. longum* subsp. *infantis* ATCC 15697 had the highest level of intrinsic H_2O_2 resistance of all strains tested and *B. animalis* subs. *lactis* BL-04 had the highest resistance among *B. lactis* strains. Inducible H_2O_2 resistance was detected in four strains, *B. longum* NCC2705, *B.* longum D2957, *B. lactis* RH-1 and *B. lactis* BL-04. Other strains showed either no

[1] Reprinted from Oberg TS, Steele JL, Ingham SC, Smeianov VV, Briczinski EP, Abdalla A, Broadbent JR. 2011. Intrinsic and inducible resistance to hydrogen peroxide in *Bifidobacterium* species. J. Ind. Microbiol. Biotechnol. 38:1947–1953 with permission (Appendix B).

difference or increased sensitivity to H_2O_2 after induction treatments. These data indicate that intrinsic and inducible resistance to hydrogen peroxide is strain specific in *B. longum* and *B. lactis* and suggest that for some strains, sublethal H_2O_2 treatments might help increase cell resistance to oxidative damage during production and storage of probiotic containing foods.

INTRODUCTION

Foods and food ingredients with "bioactive" properties, which are defined by their ability to impact human health in a manner not based solely on their nutritional value, have increased in popularity among consumers in the last decade. One example involves "probiotic" bacteria, which are "living organisms that, when ingested at sufficient numbers, exert a beneficial effect on the host organism beyond inherent general nutrition" (12). Currently, species of *Lactobacillus* and *Bifidobacterium* are the most widely used probiotic bacteria added to commercial bioactive products (33). Many species of bifidobacteria are used as probiotics, but two of the most important commercial species are *Bifidobacterium longum* and *Bifidobacterium animalis* ssp. *lactis* (henceforth described as *B. lactis*).

Although bifidobacteria are relatively minor components of the normal gastrointestinal (GI) microbiota in human adults, research indicates some strains can promote or provide several health related functions, including host resistance to infectious microbes, anti-carcinogenic activities, and improved nutritional efficiency (2, 41). Moreover, certain species of bifidobacteria are major components of the GI

microbiota in healthy, breast-fed infants, and recent work suggests the composition of GI microbiota in infants and children may influence the development of diarrheal, inflammatory, and allergic diseases (31).

No conclusive data are available on the minimal effective dose of probiotics in humans, but results from several clinical trials suggest a direct dose-effect correlation (23, 26, 35). As a result, the current WHO definition of probiotics emphasizes the need for administration of the probiotics in "adequate amounts" (27). Thus, successful application of probiotic bifidobacteria in foods is not only dependent upon the functionality of the strain, but also on the development of technologies to ensure their survival in high numbers during food processing and maintaining those high numbers during storage.

Challenges associated with probiotic delivery are exacerbated by the fact that these cells are commonly exposed to unfavorable environmental conditions during the manufacture or storage of most food-based delivery systems for bifidobacteria (29). For example, efforts to secure and maintain high numbers of viable bifidobacteria in bioactive food products are commonly impeded by the intrinsic and extrinsic properties of the food such as dehydration (low a_w), high or low temperature, low pH, high sodium chloride levels, or presence of oxygen, all of which may be deleterious to bifidobacteria (7, 9, 29, 39). To address this problem, processors may employ very large inocula or add specific growth promoters or protectants (25, 29, 44). Additionally, the ability of bacteria to resist environmental extremes is generally affected by growth phase, with stationary-phase cells showing

far greater resistance than mid-log-phase cells (45). As a result, industrial production of probiotic cultures is typically performed in large fermenters under rigid pH and temperature control, and cells are harvested at late-log or early stationary-phase growth to maximize cell biomass and vigor (22, 30).

Oxygen toxicity results from cell exposure to activated oxygen compounds such as superoxide, hydrogen peroxide (H_2O_2), and hydroxyl radicals, which induce peptide breaks, oxidation of sulfhydryl groups in proteins and oxidation of membrane lipids (6). Although H_2O_2 is the most stable of these molecules, dissociation and interaction with cellular components can form organic peroxides, which can initiate a chain reaction of oxidation (13). Most bifidobacteria organisms lack genes for catalase and superoxide dismutase, which are used by many bacteria to detoxify H_2O_2 and superoxide, respectively. Nonetheless, *Bifiobacterium* species have been shown to produce inhibitory levels of H_2O_2 when incubated in the presence of oxygen (10). Moreover, bifidobacteria are commonly added to yogurt products and the starter cultures used to manufacture yogurt, *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus* have also been shown to produce inhibitory concentrations of H_2O_2 when grown under conditions such as those encountered during bioactive food production and storage (40).

Several studies have shown environmental stress resistance in many microorganisms, including some bifidobacteria, may be dramatically improved by deliberate induction of an adaptive or inducible stress response (4, 5, 8, 11, 15, 22, 30, 37, 42). These inducible stress responses are characterized by the transient

induction of genes that encode general and specific stress proteins (e.g. chaperones and ATP-dependent proteases) and corresponding regulatory proteins (3, 16, 21, 32, 34, 46, 47).

The knowledge that sublethal stress treatments can promote cell robustness is already exploited in the manufacture and use of probiotic cultures (39), but a more detailed understanding of environmental adaptation by bifidobacteria to oxidative stress would likely reveal new strategies to improve the industrial stability, performance, and utility of these probiotics. Thus, the purpose of this research was to investigate the intrinsic and inducible H_2O_2 stress resistance in several industrially important strains of *B. longum* and *B. lactis*.

MATERIALS AND METHODS

Bacterial strains and culture conditions

Strains of *B. longum* and *B. lactis* selected for use in this study are listed in Table 3-1. Strains were maintained as glycerol freezer stocks at -80°C, and working cultures were prepared by two successive transfers (1% innoculum, v/v) into peptonized milk media (MP5) (3% proteose peptone, 1.4% glucose, 1.7% yeast extract, 0.1% Tween 80, 0.45% sodium chloride, 0.05% cysteine HCl) with anaerobic incubation at 37°C for 18 h.

Batch cultures of each strain were prepared for H_2O_2 resistance studies by a 1% (v/v) inoculation of working cultures, diluted to an OD_{600} of 1.0 in MP5 media, into one liter of MP5 media in a 1 L New Brunswick BioFlo III fermenter (New

Brunswik Scientific, Edison, New Jersey), with an agitation rate of 100 rpm and an incubation temperature of 37°C. A gas mixture of 5% carbon dioxide and 95% nitrogen was continuously passed over the headspace of the fermenter to achieve anaerobic conditions, and the pH was maintained at 6.5 by automatic addition of 15% (v/v) ammonium hydroxide. The cultures were incubated until the cells reached early stationary-phase (approximately 12 h for the *B. lactis* strains and 14 h for the *B. longum* strains).

Table 3-1 Bifidobacteria selected for this study.

Species and strain	Description (reference)
B. animalis ssp*. lactis*	
BL-04	Industrial probiotic strain [3]
DSM 10140	Industrial probiotic and type strain [3]
RH-1	Industrial probiotic strain
B. longum	
NCC2705	Industrial probiotic strain; isolated from human infant [36]
D2957	Industrial probiotic strain
B. longum ssp. *infantis*	
ATCC 15697	Industrial probiotic strain; isolated from human infant [38]

Intrinsic hydrogen peroxide resistance

Intrinsic H_2O_2 resistance of each culture was measured in 10 mL MP5 medium with addition of H_2O_2 at concentrations of 0.65 mM, 1.3 mM, 2.25 mM, 5.25 mM, and 10.5 mM, plus a control, which contained no H_2O_2. Early stationary phase cells were grown in batch culture as described, then collected by centrifugation at 3500x*g* and diluted 1:100 to obtain a cell concentration of 10^4 – 10^6 colony forming units (CFU)/mL. The cells were inoculated at 1% (v/v) into MP5 with different H_2O_2 concentrations and placed in an anaerobic jar (BD, Franklin Lakes, NJ, USA). The jar headspace was flushed with a mixture of 5% carbon dioxide/95% hydrogen then placed at 37°C. Aliquots (1 mL) were collected every hour for 6 h starting at time 0, serially diluted in sterile 0.1% peptone, and plated on MRS (Difco, Sparks, MD, USA) agar plates supplemented with 0.05% filter sterilized cysteine (MRS+C) using the spread plate technique. Agar plates were incubated in anaerobic jars at 37°C for 48 h before enumeration. Replicates were performed in quadruplicate. After each sampling time, the tubes were placed back in an anaerobic jar and the headspace was flushed with gas as described. The H_2O_2 concentration of each MP5 tube was assayed at time 0 using the SensoLyte ADHP hydrogen peroxide colorimetric assay kit (AnaSpec, San Jose, CA, USA) following the manufacturer's protocol. All H_2O_2 assays were performed in quadruplicate using optical grade removable strip 96 well plates (Thermo Scientific, Vantaa, Finland) and absorbance at 576 nm was read on a Spectramax Plus 384 plate reader (Molecular Devices Corp., Sunnyvale, CA). To standardize the results, the measured peroxide concentration before inoculation

and the CFU/mL after 1 h (for each strain) were fitted into a least squares linear regression model with the 95% confidence interval of the slopes used to determine significant differences between the strains (14).

For inducible stress response testing, lethal stress treatments for each strain were defined as the minimum H_2O_2 concentration at which there were no recoverable cells over the 6-h incubation period. Sublethal stress treatments for each strain were defined as the highest H_2O_2 concentration that resulted in no more than a 1 $\log_{10}$ decrease in cell numbers during the 6-h exposure (30).

Screening for inducible H_2O_2 stress resistance

Cells were grown to early stationary-phase in batch culture, then 10 mL samples were collected and centrifuged at 3500 x g for 5 min. The cell pellet was suspended in MP5 media warmed to 37°C that contained a sublethal H_2O_2 concentration (1.25 mM for all strains) and incubated at 20 or 60 min at 37°C in an anaerobic jar flushed with a 5% carbon dioxide/95% hydrogen gas mixture. After the 20- or 60-min sublethal H_2O_2 exposure, the cells were collected by centrifugation, and suspended in 10 mL of MP5 media warmed to 37°C that contained either 2.55 mM and 5.25 mM H_2O_2 as the lethal challenge and incubated anaerobically at 37°C. Samples (1 mL) were taken after 0, 15, and 30 min of exposure, and plated as described for intrinsic resistance studies. Controls were prepared the same way as test cultures, except that no H_2O_2 was added to the MP5 media used for the 20- or 60-min incubations prior to lethal H_2O_2 exposure. Replicates were performed in quadruplicate. Any difference in a strain's ability to

withstand a particular lethal stress treatment after a sublethal H_2O_2 exposure was expressed as a percent survival, which was calculated by dividing the $\log_{10}$ CFU/mL of surviving cells after a 30 min lethal H_2O_2 challenge by the $\log_{10}$ CFU/mL of cells after a 0 min lethal H_2O_2 exposure. To determine if the calculated percent survival was significant, means from the induced strain were compared to control means using a one-tailed two-sample *t* test without pooled variance with a = 0.05 (14).

RESULTS

Assays for intrinsic resistance to H_2O_2 exposure among commercial strains of *B. longum* and *B. lactis* showed *B. longum* ssp. *infantis* ATCC 15697 had significantly higher ($p<0.05$) intrinsic H_2O_2 resistance than all the strains tested (Figure 3-1). *B. lactis* BL-04, *B. longum* NCC2705, and *B. lactis* RH-1 showed an intermediate level of intrinsic H_2O_2 resistance, while *B. longum* D2957 and *B. lactis* DSM10140 showed the lowest intrinsic resistance to H_2O_2. The $\log_{10}$ CFU data collected each hour over the 6 h incubation was used to define the sublethal and lethal H_2O_2 concentrations for induction and challenge (Figure 3-2). The graphs in figure 3-2 show a clear division among the concentrations of H_2O_2 tested and based on the data, the sublethal concentration was determined to be 1.25mM and the lethal concentrations were determined to be 2.55mM and 5.25mM for all strains. Experiments to screen *B. longum* and *B. lactis* strains for inducible H_2O_2 stress resistance revealed most

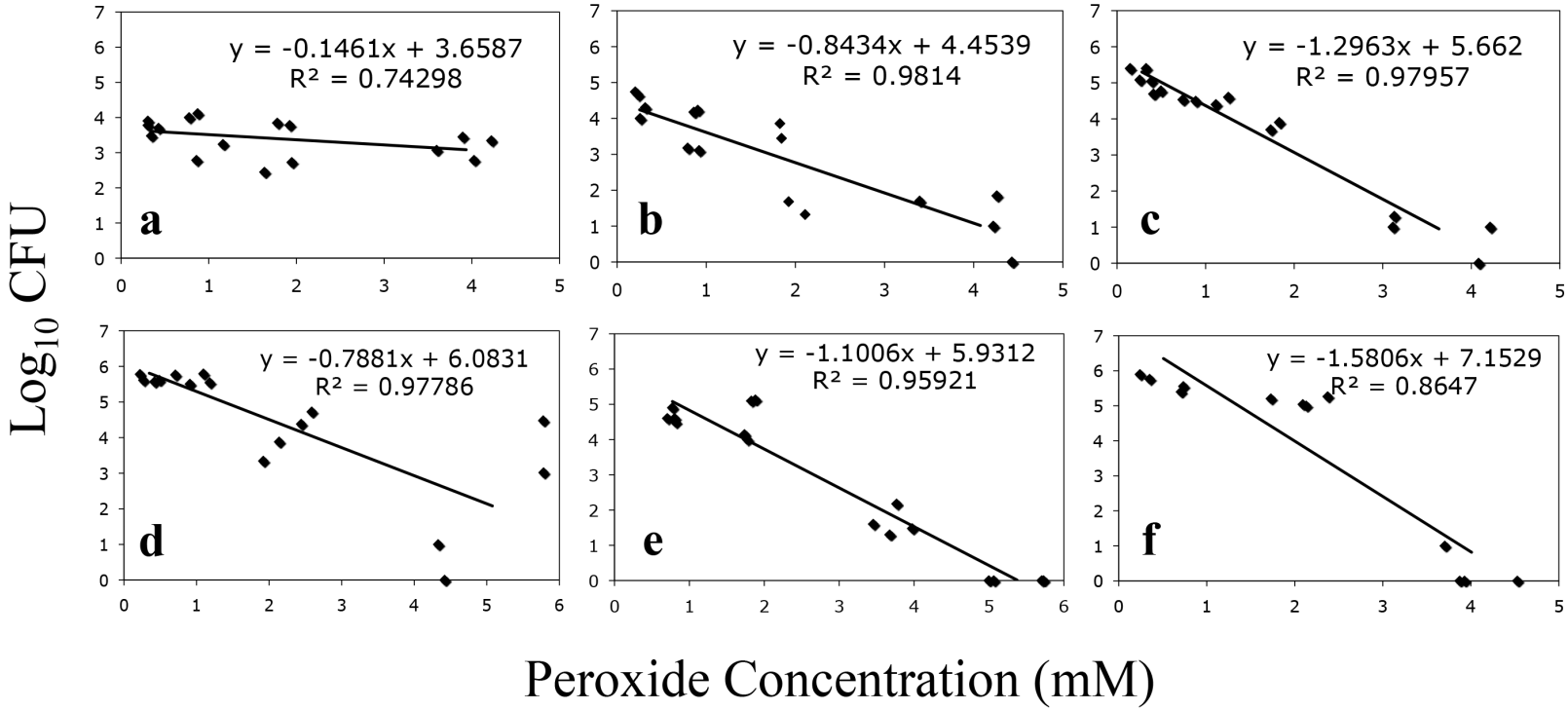

Fig. 3-1 Linear regression plots of intrinsic H_2O_2 resistance. *X-axis*, measured H_2O_2 concentration of media before inoculation. *Y-axis*, $\log_{10}$ CFU/mL after 1 h incubation. *Graphs:* **a.** *B. infantis* ATCC 15697; **b.** *B. longum* NCC2705; **c.** *B. longum* D2957; **d.** *B. lactis* BL-04; **e.** *B. lactis* RH-1; **f.** *B. lactis* DSM10140

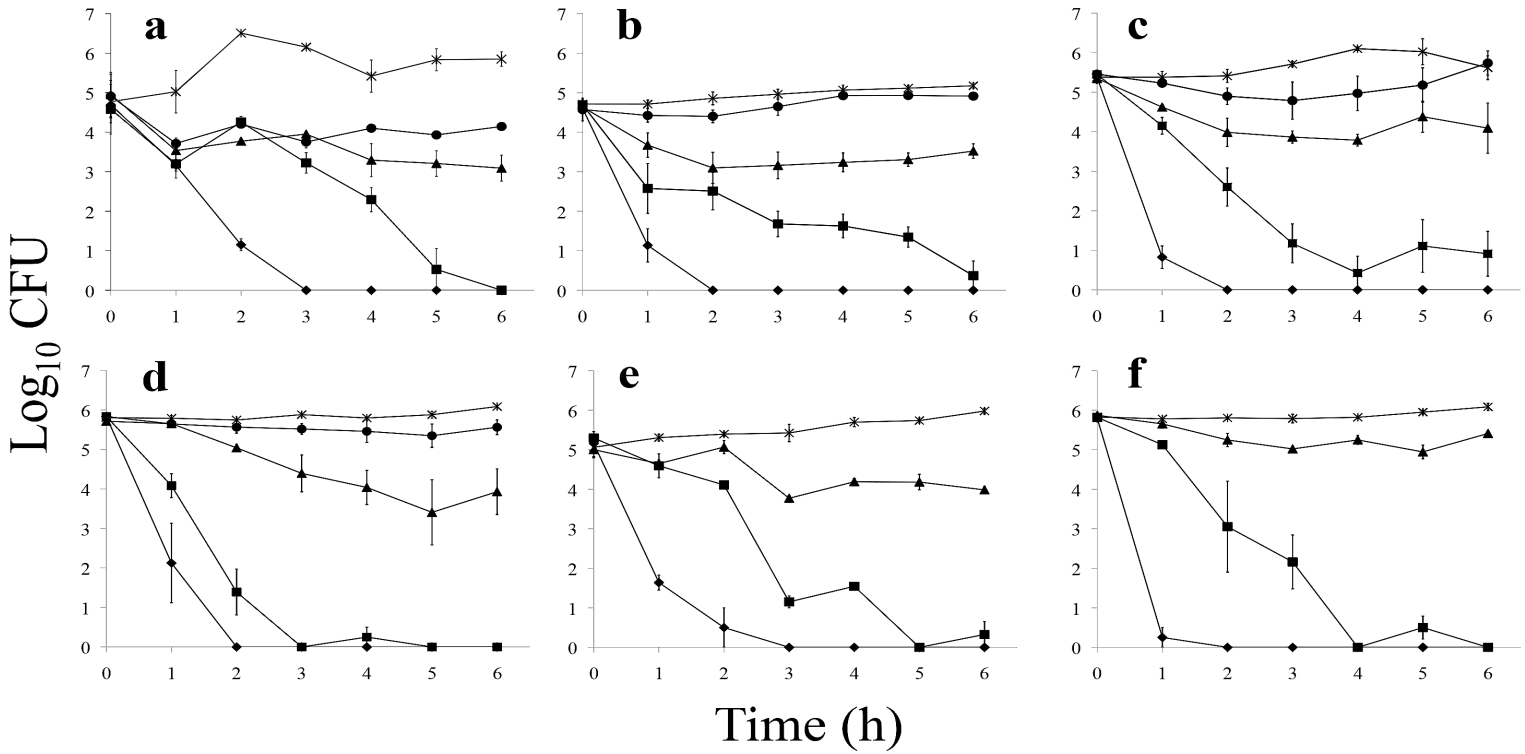

Fig. 3-2 Graphs of intrinsic H_2O_2 resistance during a 6 h exposure. *X-axis*, time (h). *Y-axis*, $\log_{10}$ CFU/mL. *Graphs:* **a.** *B. infantis* ATCC 15697; **b.** *B. longum* NCC2705; **c.** *B. longum* D2957; **d.** *B. lactis* BL-04; **e.** *B. lactis* RH-1; **f.** *B. lactis* DSM10140. *Peroxide concentrations:* cross (×), Control; filled circle (●), 0.66mM H_2O_2; filled triangle (▲), 1.25mM H_2O_2; filled square (■), 2.25mM H_2O_2; filled diamond (u), 5.25mM H_2O_2. *Error bars* correspond to the standard error of the mean (SEM).

strains displayed a decreased percent survival after the lethal challenge compared to control cells (Figure 3-3). These results show that some strains were unable to mount an inducible stress response under the conditions tested, and in some cases, cells that were exposed to sublethal H_2O_2 were more sensitive to lethal H_2O_2 concentrations than control cells. However, 60 min sublethal H_2O_2 treatment with *B. longum* NCC2705 significantly ($p<0.05$) increased the survival of this strain at both lethal H_2O_2 concentrations tested (Figure 3-4), and a significant ($p<0.05$) increase in survival was also recorded for *B. longum* NCC2705 and *B. longum* D2957 given a 20 min treatment followed by a lethal challenge at 2.55 mM H_2O_2 (Figure 3-3A). Among the *B. lactis* strains, *B. lactis* RH-1 and *B. lactis* BL-04 cells given a 20 min induction treatment showed a significant ($p<0.05$) increase in survival after 5.25 mM H_2O_2 challenge (Figure 3-4B).

DISCUSSION

Results from this study indicate that intrinsic and inducible H_2O_2 resistance is both species and strain specific in *B. longum* and *B. lactis*. Previous studies have investigated H_2O_2 resistance in bifidobacteria and LAB at a wide range of H_2O_2 concentrations under static conditions in differing buffer solutions (17, 18, 19, 20, 28, 43). However, our study design sought to explore H_2O_2 resistance of each strain in a milk peptone-based growth medium similar to those used for commercial production of probiotic cultures instead of buffer, so that cells had an opportunity for active metabolism during exposure. Because H_2O_2 is a strong oxidizing agent, we

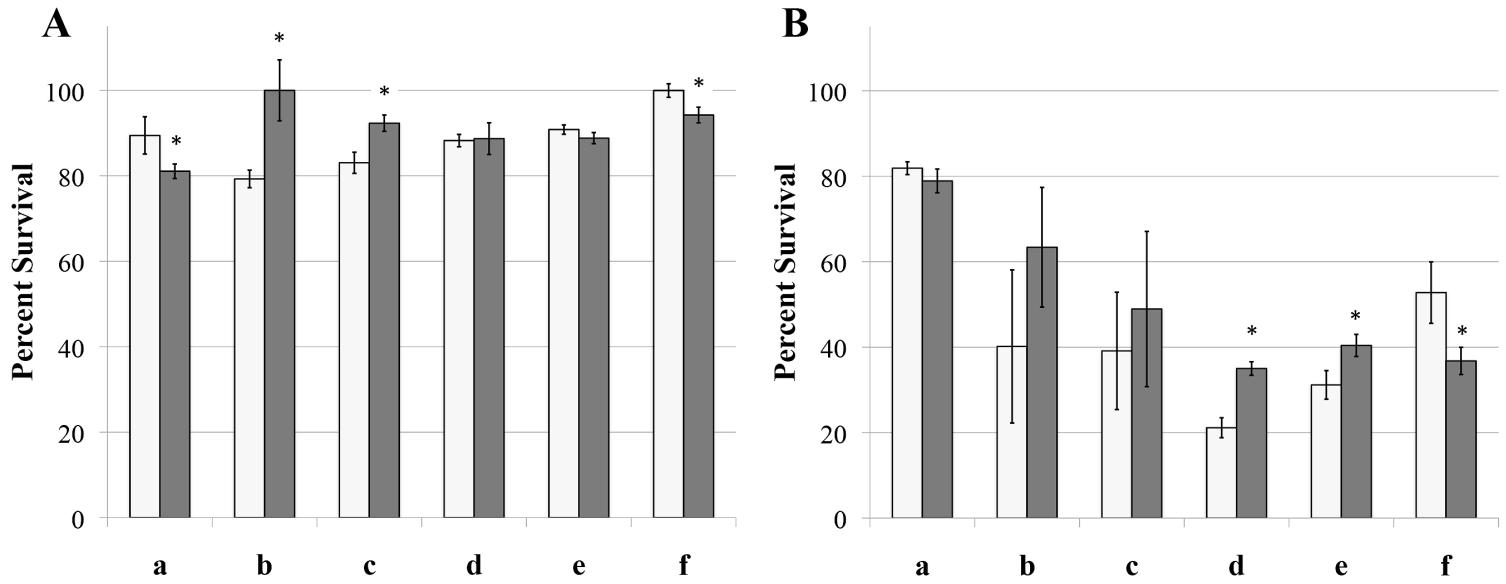

Fig. 3-3 Percent survival of *Bifidobacterium* after a 20 min experimental stress response induction at 1.25 mM H_2O_2 challenged at lethal concentrations of **A** 2.55 mM H_2O_2 and **B** 5.25 mM H_2O_2. *Lanes:* **a** *B. infantis* ATCC 15697; **b** *B. longum* NCC2705; **c** *B. longum* D2957; **d** *B. lactis* BL-04; **e** *B. lactis* RH-1; **f** *B. lactis* DSM10140. *Open square*, Control; *filled square,* Induced. Each value is the mean of four replicates. *Error bars* correspond to the standard error of the mean (SEM). *Asterisk* (*) denotes bars that have a mean percent survival significantly different ($p<0.05$) from control.

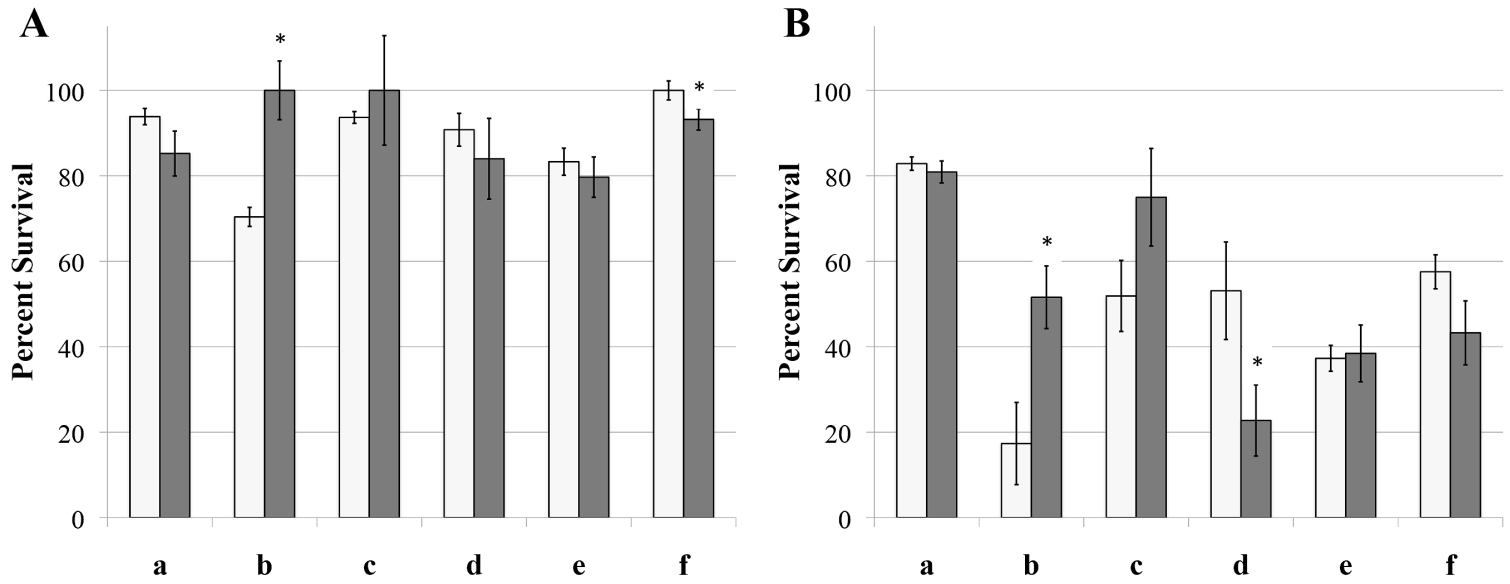

Fig. 3-4 Percent survival of *Bifidobacterium* after 60 min experimental stress response induction at 1.25 mM H_2O_2 challenged at lethal concentrations of **A** 2.55 mM H_2O_2 and **B** 5.25 mM H_2O_2. *Lanes:* **a** *B. infantis* ATCC 15697; **b** *B. longum* NCC2705; **c** *B. longum* D2957; **d** *B. lactis* BL-04; **e** *B. lactis* RH-1; **f** *B. lactis* DSM10140. *Open square*, Control; *filled square,* Induced. Each value is the mean of four replicates. *Error bars* correspond to the standard error of the mean (SEM). *Asterisk* (*) denotes bars that have a mean percent survival significantly different ($p<0.05$) from control.

anticipated there would be a loss in concentration upon addition to broth medium and preliminary tests confirmed the concentration of H_2O_2 decreased from 20-60% corresponds to H_2O_2 resistance -similar to Z-value determination in thermal destruction of an organism (24), and a steeper slope shows faster cell death at increasing concentrations of H_2O_2.

Comparison of the whole genome sequences for *B. lactis* DSM10140, *B. lactis* BL-04, *B. longum* NCC2705, and *B. infantis* 15697 showed that all of the strains lacked genes for the classical oxidative stress response enzymes superoxide dismutase, catalase, and a true peroxidase (3, 38). Further genetic analysis of these strains reveals that they contain genes for flavin proteins, although they lack a gene for flavin reductase, as well as genes for a thioredoxin reductase-thioredoxin system and a peroxiredoxin. The thioredoxin reductase systems works in conjunction with NADPH to maintain the redox potential in the cell for proper disulfide bond formation in proteins, and serves as an electron donor for enzymes such as ribonucleotide reductase (1). This system also donates electrons to peroxiredoxin for the reduction of H_2O_2 to H_2O. Interestingly, comparison of the whole genome sequence of *B. lactis* BL-04 and *B. lactis* DSM10140 shows that they are almost identical (3). Our data show that there are large differences between the intrinsic H_2O_2 resistance and inducible H_2O_2 stress responses of these strains, which should be attributable to one or more of their minor genetic differences. Further research is underway to explore this observation.

Although certain strains showed higher H_2O_2 resistance than others, the lethal H_2O_2 concentration for all strains was relatively low (2.55-5.25 mM). These values are within the concentration range of H_2O_2 produced by lactic starter cultures during manufacture of yogurt and other bioactive foods (40), which underscores the need for technologies to enhance H_2O_2 resistance in bifidobacteria. Our results suggest that a sublethal H_2O_2 exposure could be used to enhance H_2O_2 resistance of some strains (e.g. *B. longum* NCC2705 and *B. lactis* BL-04), and increase their survival in functional foods. Additionally, more detailed studies of inducible H_2O_2 stress resistance in these strains may reveal strategies to enhance H_2O_2 resistance in a broader range of strains.

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CHAPTER 4[1]

IDENTIFICATION OF PLASMALOGENS IN THE CYTOPLASMIC MEMBRANE OF *BIFIDOBACTERIUM ANIMALIS* SUBSP. *LACTIS*

ABSTRACT

Plasmalogens are ether-linked lipids that may influence oxidative stress resistance of eukaryotic cell membranes. Since bacterial membrane composition can influence environmental stress resistance, we explored the prevalence of plasmalogens in the cytoplasmic membrane of *Bifidobacterium animalis* subsp. *lactis*. Results showed plasmalogens are a major component of the *B. animalis* subsp. *lactis* membrane.

INTRODUCTION

Evidence suggests several species of bifidobacteria have probiotic properties (20), and two commercially important species are *Bifidobacterium longum* and *Bifidobacterium animalis* ssp. *lactis*. One of the challenges associated with use of bifidobacteria probiotics involves the loss of viability due to the unfavorable environmental conditions that are encountered during the manufacture and storage of most food-based delivery systems (21). One potential mechanism to enhance cell survival involves manipulation of bacterial cell membrane fatty acid (CMFA)

[1] Reprinted from Oberg TS, Ward RE, Steele JL, Broadbent JR. 2012. Identification of plasmalogens in the cytoplasmic membrane of *Bifidobacterium animalis* subsp. *lactis*. Appl. Environ. Microbio. **78**:880–884 with permission (Appendix B).

composition (14, 22). Several studies have shown that CMFA composition influences membrane fluidity, proton permeability and the activity of a variety of transport proteins (8, 12, 23, 28). Cell exposure to acidic pH, for example, can trigger an increase in the level of saturated, branched, or cyclopropane fatty acids in the cell membrane (2, 4, 6, 9, 11, 13). These changes render the cell membrane more rigid and result in greater cell membrane stability in acidic environments. Among bifidobacteria, a bile salt resistant *B. animalis* subsp. *lactis* mutant has been shown to increase the unsaturated/saturated fatty acid ratio of its CMFA during bile salt exposure whereas wild-type cells showed a decrease in the unsaturated/saturated fatty acid ratio (22). This study also showed that in both strains there was a large decrease in the amount of C19:0 cyclopropyl fatty acid in response to bile salt exposure.

Plasmalogens are phospholipids that contain a vinyl ether bond at the SN1 position as opposed to an ester bond, and display distinct physical properties compared to diacyl analogs. Plasmalogens are widespread among eukaryotes, accounting for up to one-fifth of the total phospholipid pool of humans (26). In eukaryotes, cells with high CMFA plasmalogen content are associated with oxidative environments, and display lower membrane ion permeability and surface potential, and an increase in cell membrane fluidity (26). Additionally, the vinyl ether bond is more easily oxidized than the carbon-carbon double bond of unsaturated fatty acids, and in contrast to oxidized unsaturated fatty acids plasmalogens do not propagate free radicals in response to peroxides (5, 17). Because of these characteristics,

plasmalogens have been proposed to act as anti-oxidants in membrane physiology via protection of unsaturated fatty acids and membrane proteins from harmful oxidation (7, 29). Several *Clostridium*, *Mycobacterium* and methanogenic archea species have also been found to possess vinyl ether-linked lipids in their membrane, but little is known about their role (10, 13, 27). Very few studies on the membrane composition of bifidobacteria have noted vinyl ether linked lipids (1), or more commonly group them with their esterified analogs (22). Because of their unique properties, plasmalogen content should be considered in research to explore the role of CMFA composition in environmental stress resistance among bifidobacteria. Here, we use a previously described and simple methodology for derivitization of plasmalogens to isolate these lipids, and provide mass spectra lacking in the literature but necessary for their identification. Results reveal plasmalogens are a significant component of the cytoplasmic membrane of *B. animalis* subsp. *lactis*.

MATERIALS AND METHODS

Two industrially important *B. animalis* subsp. *lactis* strains, DSM10140 and BL-04 (3), were maintained as glycerol freezer stocks at -80^{o}C, and working cultures were prepared by two successive transfers (1% innoculum, vol/vol) into peptonized milk medium (MP5) (19) and incubated at 37^{o}C for 18 h in anaerobic chambers (Becton Dickinson Microbiology Systems, Cockeysville, MD.). Batch cultures of each strain were prepared by dilution of the working culture to an absorbance at 600 nm (A_{600}) of 1.0 in MP5 medium, then inoculated at 1% (vol/vol) into 1 L of MP5 in a

New Brunswick BioFlo III fermenter (New Brunswik Scientific, Edison, NJ), and finally incubated at 37°C with an agitation rate of 100 rpm to prevent sedimentation. A gas mixture of 5% CO_2 and 95% N_2 was continuously passed over the headspace of the fermenter to achieve anaerobic conditions, and the pH was maintained at 6.5 by automatic addition of 15% (vol/vol) NH_4OH. The cultures were incubated until the cells reached early stationary-phase (approximately 12 h) (19).

Twenty mL of cells were centrifuged and washed twice with phosphate buffered saline (PBS). Total fatty acids were extracted from cell pellets by acid hydrolysis and methylation according to the MIDI laboratory protocol described by Sasser (25). To inhibit oxidation, butylated hydroxytoluene was added to each sample, and the GC vial headspace was flushed with nitrogen gas. The samples were then analyzed on a GCMS-QP2010S (Shimadzu Scientific Instruments, Columbia, MD) equipped with a flame ionization detector and fitted with a 10-m guard column and a 30-m DB5 capillary column. The injector temperature was held at 250°C and 1µL of the sample was injected splitless. The temperature of the oven was held at 50°C for 1 min then increased to 150°C at 20°C/min, and then increased to 250°C at 4°C/min, with the final temperature of 250°C held for 1 min. Helium was used as the carrier gas at a column flow rate of 1.79 mL/min. Electron impact ionization at 70 eV was used for fragmentation. A bacterial acid methyl esters standards mix (SUPELCO, Bellefonte, PA) was used to identify derivatized methyl esters in the samples. The data were normalized and the percent of total for each fatty acid was

determined. Because ion fingerprints for plasmalogens are not available in the standard or the literature, samples containing suspected plasmalogens were collected as described, and sent to the University of California Riverside Analytical Chemistry Instrumentation Facility for GC-accurate mass of ionized lipid fragments. Samples were also sent to the University of Utah Mass Spectrometry and Proteomics Core Facility for precise mass measurements of the intact parent species using GC-electrospray ionization accurate mass on a Waters GCT Premiere Lockmass.

RESULTS AND DISCUSSION

The vinyl ether bond of plasmalogens is easily hydrolyzed under acidic conditions to form an aldehyde, which in the presence of methanol rapidly reacts to form dimethyl-acetals (DMA) (Figure 4-1) (16). The isolated DMA elute at a slower rate on the GC column than their methyl ester analogs, allowing separation. Figure 4-2 shows a chromatogram of the membrane fatty acids of *B. animalis* subsp. *lactis* DSM10140. After analysis and comparison to the standard, peaks of interest were identified based on their elution time and the presence of a strong ion peak of 75. Under electron impact ionization, DMA fragments extensively, with the most abundant ion (m/z = 75) resulting from the loss of the DMA head-group. This ion peak is the principal identifier of DMA and is unique to these molecules (27). The extracted mass spectrum of each peak (Figure 4-3) was analyzed to determine its empirical formula.

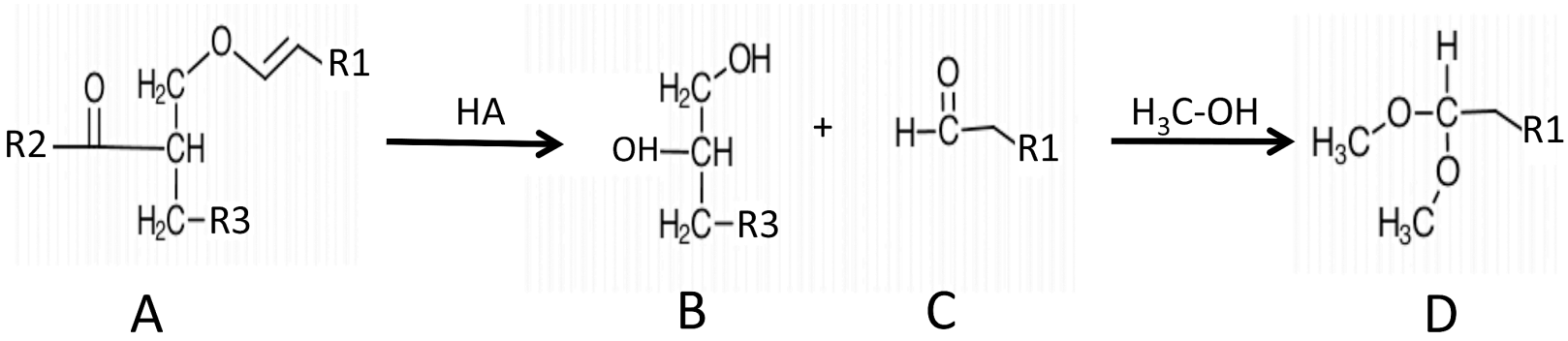

FIG 4-1 Acid hydrolysis of plasmalogens and derivatization to dimethyl-acetals. A, Plasmalogen; B, Phosphoglycerol; C, Fatty aldehyde; D, Dimethyl-acetal. HA, Bronsted acid; R1/R2, fatty acid carbon tail; R3, phospholipid head group.

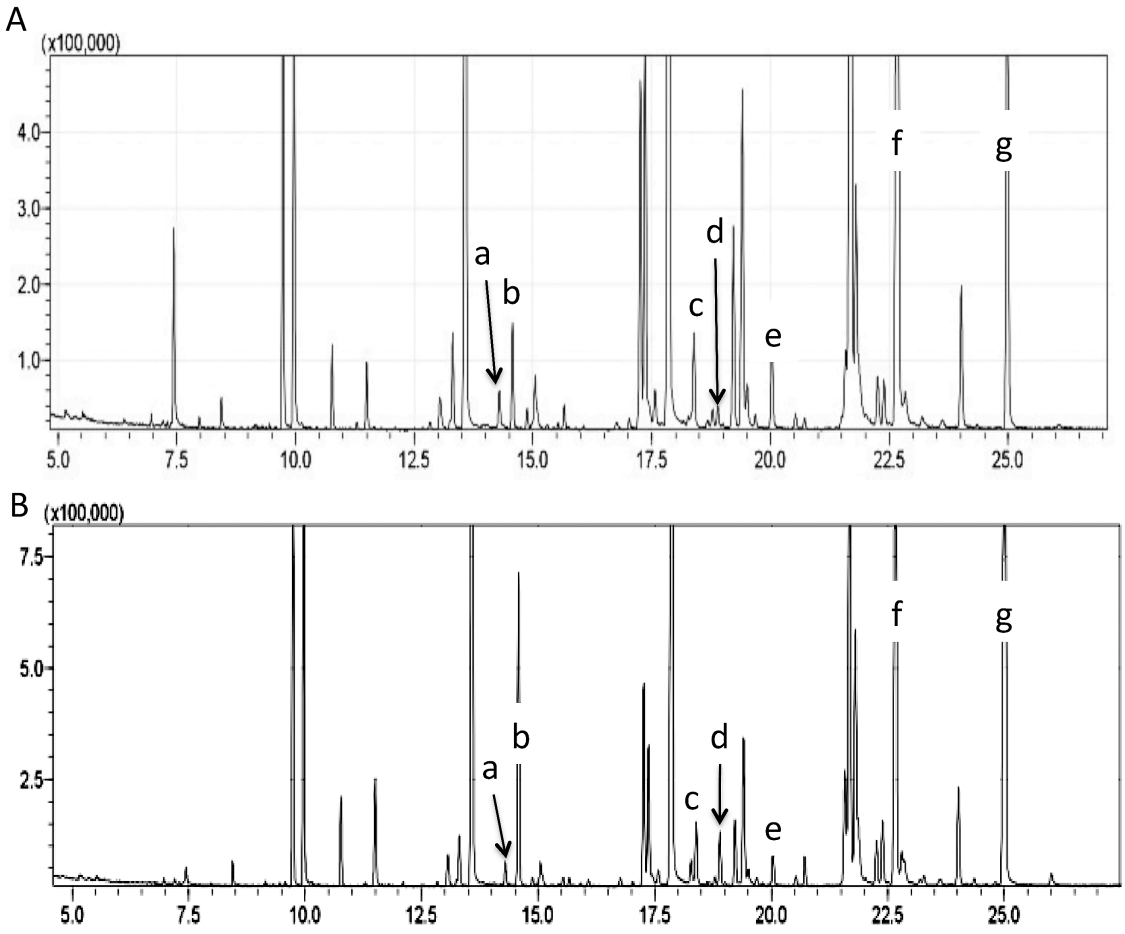

FIG 4-2 Gas chromatogram of cytoplasmic membrane lipid extract from *Bifidobacterium animalis* subsp. *lactis* DSM10140 (A) and *Bifidobacterium animalis* subsp. *lactis* BL-04 (B) showing peaks for dimethyl-acetal derived plasmalogens. Peaks of interest include C14:1 (a), C14:0 (b), C16:1 (c), C16:0 (d), C17:0 cyclopropyl (e), C18:1 (f) and C19:0 cyclopropyl (g). *y* axis, signal intensity; *x* axis, time (min).

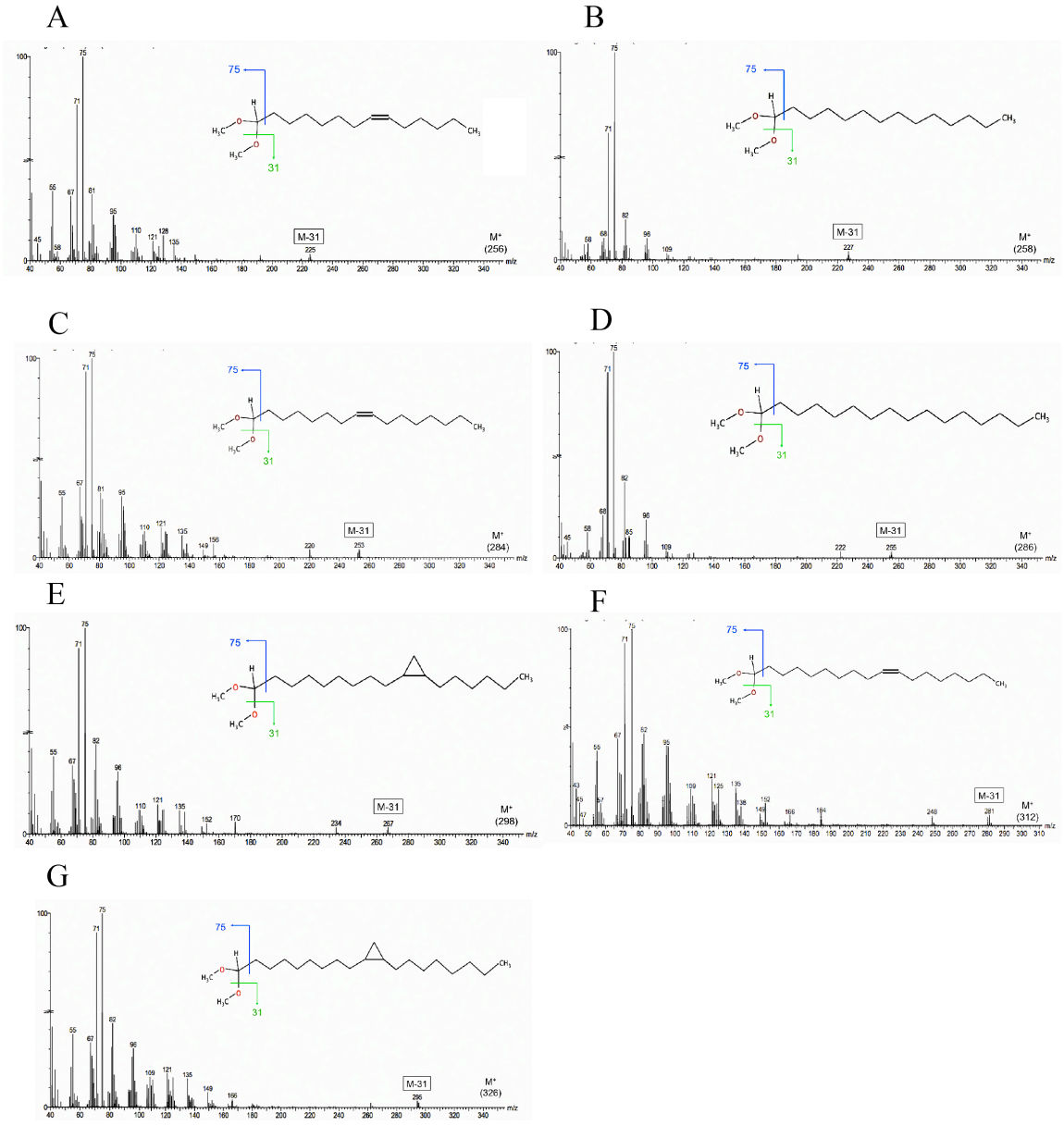

FIG 4-3 Mass spectra of dimethyl-acetal derived plasmalogens. Spectra are shown for C14:1 (A), C14:0 (B), C16:1 (C), C16:0 (D), C17:0 cyclopropyl (E), C18:1 (F) and C19:0 cyclopropyl (G). *y* axis, relative abundance; *x* axis, mass-to-charge ration *m/z*.

Another important fragmentation product is the parent ion molecular mass minus 31 (M-31) that results from loss of a methoxy group (Figure 4-1). These peaks reveal the number of carbons and level of saturation in the alkenyl moieties of the DMA. Table 4-1 shows the measured accurate mass mass to charge ratio (*m/z)* of M-31 for each peak and compares them to the calculated *m/z*. A parts-per-million (ppm) error was calculated for each *m/z*, with each error under the threshold value for significance (5 ppm). Under electron impact ionization, these molecules fragment easily and do not produce a detectable parent ion. Electrospray ionization was used to measure the *m/z* of the parent ion peak. This is a soft ionization method which allows for the addition of an electron without fragmenting the molecule. The measured accurate *m/z* of the parent ion peaks were also compared to the calculated *m/z*, with all having a ppm error less than 5 (Table 4-1). Together, these mass spectrometry (MS) data provide positive identification of the DMA in *B. animalis* subsp. *lactis* membrane samples.

Previous research in our laboratory (19) showed *B. animalis* subsp. *lactis* BL-04 has significantly greater intrinsic resistance to H_2O_2 than strain DSM10140. Data collected in this study show plasmalogens make up a significant proportion of the total membrane composition of *B. animalis* subsp. *lactis* DSM10140 and BL-04 (26.34% ± 4.73% and 30.35% ± 5.21%, respectively). Although the amount of plasmalogens in DSM10140 and BL-04 as a percent of total CMFA are not significantly different (P = 0.05), levels of C19:0 cyclopropyl vinyl ether lipids are

significantly higher ($P < 0.05$) in strain BL-04 (15.48% ± 7.01%) versus DSM10140 (6.71% ± 1.74%).

TABLE 4-1 Measured masses of DMA-derived plasmalogens.

		M -31 Peaks		Parent ion peaks	
Fatty acid	Empirical formula	Calculated *m/z*	Measured *m/z*	Calculated *m/z*	Measured *m/z*
C14:1	$C_{16}H_{32}O_2$	225.2062	225.2216[a]	256.2402	256.2396[a]
C14:0	$C_{16}H_{34}O_2$	227.2219	227.2373[a]	258.2559	258.2481[a]
C16:1	$C_{18}H_{36}O_2$	253.2375	253.2547[a]	284.2715	284.2697[a]
C16:0	$C_{18}H_{38}O_2$	255.2532	255.2679[a]	286.2872	286.3019[a]
C17:0 cyclopropyl	$C_{19}H_{38}O_2$	267.4760	267.2675[a]	298.5100	298.3925[a]
C18:1	$C_{20}H_{40}O_2$	281.2688	281.2837[a]	312.3028	312.3028[a]
C19:0 cyclopropyl	$C_{21}H_{42}O_2$	295.2845	295.2551[a]	326.3185	326.3138[a]

[a]Measured mass with error less than 5 ppm.

A previous study (1) reported that oxygen-tolerant fecal isolates of *Bifidobacterium* had a high content of plasmalogens in the CMFA, and data from that work also support a direct correlation between oxygen tolerance and higher CMFA concentrations of C19:0 cyclopropyl plasmalogens. Because plasmalogens have been shown to have physical attributes that affect membrane physiology differently compared to the ester-linked analogs, it is important to consider these lipids when characterizing the membrane composition of bifidobacteria. The high concentrations of plasmalogens in *B. animalis* subsp. *lactis* membranes, together

with strain-specific differences in lipid species correlated with H_2O_2 sensitivity, suggest these lipids may play an important role in environmental stress resistance. Further study is required to determine and understand the role of plasmalogens in membrane physiology and environmental stress adaptation of bifidobacteria.

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CHAPTER 5[1]

GENETIC AND PHYSIOLOGICAL RESPONSES OF *BIFIDOBACTERIUM ANIMALIS* SUBSP. *LACTIS* TO HYDROGEN PEROXIDE STRESS

ABSTRACT

Consumer interest in probiotic bifidobacteria is increasing, but industry efforts to secure high cell viability in foods is undermined by the sensitivity of these anaerobes to oxidative stress. To address this limitation, we investigated genetic and physiological responses of two fully sequenced *Bifidobacterium animalis* subsp. *lactis* strains, BL-04 and DSM 10140, to hydrogen peroxide (H_2O_2) stress. Although the genome sequences for these strains are highly clonal, prior work showed they differ in both intrinsic and inducible H_2O_2 resistance. Transcriptome analysis of early stationary phase cells exposed to a sub-lethal H_2O_2 concentration detected significant ($P<0.05$) changes in expression of 138 genes in strain BL-04 after 5 min, and 27 genes after 20 min. Surprisingly, no significant changes in gene expression were detected in strain DSM 10140 at either time. Genomic data suggested differences in H_2O_2 stress resistance might be due to a mutation in a BL-04 gene encoding long chain fatty acid-coenzyme A (CoA) ligase. To explore this possibility, membrane fatty acids were isolated and analyzed by gas chromatography-mass spectrometry (GC-MS). Results confirmed the strains had significantly different

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[1] Reprintted from Oberg TS, Ward RE, Steele JL, Broadbent JR. 2013. Genetic and physiological responses of *Bifidobacterium animalis* subsp. *lactis* to hydrogen peroxide stress. J. Bacteriol. **195**:3743–3751 with permission (Appendix B).

lipid profiles; the BL-04 membrane contained higher percentages of $C_{14:0}$ and $C_{16:0}$, and lower percentages of $C1_{6:1n7}$ and $C_{18:1n9}$. Alteration of the DSM 10140 membrane lipid composition using modified growth medium to more closely mimic that of BL-04 yielded cells that showed increased intrinsic resistance to lethal H_2O_2 challenge, but still did not display an inducible H_2O_2 stress response. Results show deliberate stress induction or membrane lipid modification can be employed to significantly improve H_2O_2 resistance in *B. animalis* subsp. *lactis* strains.

INTRODUCTION

Bifidobacteria are Gram-positive rods of irregular shape with a G+C content of 55-67%, and are part of the normal gastrointestinal flora in human infants and adults (1, 2). Bifidobacteria have been associated with several health related benefits, including a decrease in severity of the side effects associated with use of antibiotics, reduced incidence of infection in patients receiving irradiation therapy, decrease in the duration of diarrhea due to various etiologies, reduced frequency of allergic reactions, and alleviation of constipation (3-8). Although no conclusive data is available on a minimal effective dose of probiotics in humans, results from clinical trials suggest a direct dose-effect correlation with probiotic efficacy (9, 10). This means bifidobacteria likely need to be consumed at very high levels ($>10^7$ cfu) in bioactive foods to effect a probiotic outcome. At present, yogurt or fermented milks are the most common foods for delivery of probiotic bifidobacteria, but their incorporation into other foods is increasing. A major hurdle to production and

storage of bioactive foods containing bifidobacteria involves the susceptibility of these cells to oxidative stress. Bifidobacteria are anaerobic, and therefore lack common enzymes for detoxification of oxidative free radicals produced in the cell, such as catalase and superoxide dismutase (11, 12, 13). However, previous research in our laboratory has demonstrated variability in the intrinsic and inducible resistance of bifidobacteria strains to hydrogen peroxide (H_2O_2) (14). The purpose of this study was to investigate the transcriptional and physiological responses of 2 closely related strains of *Bifidobacterium animalis* subsp. *lactis* (*B. animalis* subsp. *lactis*), BL-04, a human fecal isolate, and DSM 10140, a strain originally isolated from French yogurt (12), to sublethal H_2O_2 exposure in an industrial growth medium. These strains were chosen based on their current use in industry as probiotics in bioactive foods, the availability of complete genome sequence information for both, and marked differences in their inducible and intrinsic H_2O_2 resistance (12, 14). Specifically, 20 min exposure to sublethal concentration (1.25 mM) of H_2O_2 was shown to significantly improve survival ($P < 0.05$) of *B. animalis* subsp. *lactis* BL-04, while survival of strain DSM 10140 was significantly decreased by this treatment (14). Additionally, *B. animalis* subsp. *lactis* BL-04 showed two-fold higher intrinsic H_2O_2 resistance than DSM 10140 (14).

MATERIALS AND METHODS

Culture conditions. Bacterial strains were maintained as glycerol freezer stocks at -80^{o}C, and working cultures were prepared by two successive transfers

(1% innoculum, [vol/vol]) into peptonized milk medium (MP5) (14) with anaerobic incubation at 37°C for 18 h. Batch cultures of each strain were prepared by dilution of the working culture to an absorbance at 600 nm (A_{600}) of 1.0 in MP5 medium, then inoculated at 1% (vol/vol) into 1 L of MP5 in a New Brunswick BioFlo III fermenter (New Brunswik Scientific, Edison, NJ), then incubated at 37°C with an agitation rate of 100 rpm to prevent sedimentation. A gas mixture of 5% CO_2 and 95% N_2 was continuously passed over the headspace of the fermenter to achieve anaerobic conditions, and the pH was maintained at 6.5 by automatic addition of 15% (vol/vol) NH_4OH. The cultures were incubated until the cells reached early stationary-phase (approximately 12 h; ~log 5.8) (14).

RNA isolation. Cells from 5 mL samples grown in the conditions indicated just above were harvested by centrifugation at 7500 x g for 10 min. The cell pellets were suspended in 50 mL of pre-warmed MP5 media containing a sublethal H_2O_2 concentration of 1.25 mM and held at 37°C for 5 (T1) or 20 (T2) min (14). Immediately after treatment, 100mL of RNAprotect bacterial reagent (Qiagen, Inc., Valencia, CA) was added to the cell suspensions to stop transcription and prevent mRNA degradation. A control sample was also prepared, which was not exposed to H_2O_2. Cells in RNAprotect were held at room temperature for 10 min, then collected by centrifugation at 9500 x *g* for 10 min and stored at -20°C until RNA isolation.

Cell pellets were thawed at room temperature and suspended in 900 µL of lysozyme solution (20 mg/mL in TE buffer) that also contained 20 U of mutanolysin (Sigma-Aldrich). Samples were incubated for 30 min at 37°C on a shaker incubator

at 240 rpm, after which 20 µl of proteinase K (Omega Bio-Tek Inc., Norcross, GA) (>600 mAU/ml) was added and the samples were returned to the shaker/incubator for 30 min. The RNA was then isolated using the Aurum total RNA mini kit (Biorad, Hurcules, CA) following the vendor's recommended procedures. The quantity of recovered RNA was measured with a NanoDrop 8000 spectrophotometer (ThermoFisher Scientific, Waltham, MA) and the quality of the RNA was assayed using an Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany). Samples that had sufficient quantities (>10µg) of quality RNA were stored at -80^{o}C until needed.

Synthesis and labeling of cDNA. cDNA was synthesized and labeled as recommended by the Affymetrix (Santa Clara, CA) protocol for prokaryotic target preparation in the GeneChip Expression Analysis Technical Manual. The cDNA was fragmented into approximately 50-100 bp using DNAse I and labeled with GeneChip DNA labeling reagent (Affymetrix, Santa Clara, CA) and terminal deoxynucleotidyl transferase (Promega, Madison, WI). Fragmentation labeling efficiency was measured by gel shift assay.

DNA microarrays. Sample hybridization was performed at the Center for Integrated Bio-systems at Utah State University against a custom Affymetrix bifidobacterial DNA microarray designed to include 1,761 shared plus unique chromosomal genes predicted to occur in *B. animalis* subsp. *lactis* BL-04 and DSM 10140 (12). The only predicted coding sequences not included in the microarray design were redundant transposases and rRNA genes. Hybridization was

performed according to the Affymetrix protocol for prokaryotic target hybridization in the GeneChip Expression Analysis Technical Manual using a hybridization temperature of 50^{o}C. The DNA microarrays were scanned using the HP GeneArray scanner (Affymetrix, Santa Clara, CA) to generate raw intensity values for each probe. Statistical analysis of microarray data was performed using Bioconductor (www.bioconductor.org) in the open source statistical platform R (www.r-project.org). The raw probe data was preprocessed using the RMA-MS method (15) and filtered to only include genes that had a high signal intensity and a low coefficient of variation. To test for differential expression, the preprocessed, filtered data was analyzed using the limma/eBayes method (16). Genes were determined to be significantly differentially expressed if they had a false discovery rate corrected P-value less than 0.05. The significantly differentially expressed genes were grouped according to function and by treatment times and strain.

Microarray validation. To validate the microarray data, quantitative real-time PCR (RT-PRC) was performed for 6 different genes (Table 5-1) using cDNA produced after each treatment as described by Smeianov et al. (17). A log-fold change (LFC) was calculated between control and treatment samples, and graphed vs. the LFC calculated from the microarray data. A positive LFC represents up-regulation of a particular gene in treated cells versus the control, while a negative LFC reflects gene down-regulation.

TABLE 5-1 Target genes oligonucleotide primers for RT-PCR.

Protein function (Gene ID)	Primer Sequence		Amplicon size (bp)	Annealing temp (^{o}C)
	Forward	Reverse		
Peroxiredoxin (Balac_0865)	CCGTGTGAAGGCGTCGCAGT	GCTCGGCTCGAGCGTTTCGT	91	61.5
Ribonucleotide Reductase (Balac_0326)	CACCACGCTCGCCGAGATCC	TGCTCATCGTGATGCGCCCG	104	61.5
Long Chain acyl-CoA synthetase (Balac_1406)	TCCAGGGCTACGGCCTGACC	CGCCGGTGGGTGAGATACGC	123	61.5
dnaK (Balac_1557)	ACGCCGCTGTCCCTCGGTAT	ACGGCTGGTTGTCTTCGGCG	121	61.5
3-oxoacyl-ACP Reductase (Balac_0317)	AAGCTCGTGCGTGACCTGGC	TGGGGTCGTTCGCGTTCGTG	94	61.5
Multidrug resistance efflux pump (Balac_1405)	TGCGTGGAAACCGGCGACTC	CCGCCCACTTCGTTCTGCGT	149	61.5

Membrane fatty acid analysis. To determine whether H_2O_2 exposure altered cytoplasmic membrane fatty acid (CMFA) composition, cells were grown in batch culture as described before, and treated with a sublethal H_2O_2 concentration of 1.25 mM for 5 (T1) or 20 (T2) min. Cells in 20 mL samples were collected by centrifugation at 5000 × *g* for 5 min, then washed twice with phosphate buffered saline. Membrane fatty acids were then isolated from the pelleted cells according to the protocol of Sasser (18) and identified using gas chromatography as described previously (19). An untreated control sample was also prepared.

To determine the effect of exogenous fatty acids in the growth medium on CMFA composition, cells were grown to early stationary phase in MP5 broth containing 1% Tween 80 ($C_{18:1\ n9}$), 1% Tween 20 ($C_{12:0}$), or no exogenous fatty acid substrate. Bacteria in 20-mL samples were collected and analyzed as described above.

Inducible and intrinsic H_2O_2 resistance. Cells were grown to early stationary phase in MP5 media with 1% Tween 80 ($C_{18:1\ n9}$) or no added exogenous fatty acids, exposed to a sublethal H_2O_2 concentration of 1.25 mM for 20 or 60 min, then challenged with a 30-min exposure to a lethal H_2O_2 concentration of 2.55 or 5.25 mM (14). Control cells were also prepared that received no H_2O_2 treatment. Samples were plated on MRS agar containing 0.05% filter sterilized cysteine after 0 and 30 mins, and then incubated anaerobically at 37°C for 48 h before enumeration. Results are expressed as a percent survival, which is calculated by dividing the $\log_{10}$ CFU/mL of surviving cells after 30 mins by the $\log_{10}$ CFU/mL of cells after 0 min

(14). The student *t* test was used to identify significant differences ($P < 0.05$) between treatment means (20).

Microarray data accession number. Microarray hybridization data have been deposited in Gene Expression Omnibus under accession number GSE44382.

RESULTS AND DISCUSSION

Influence of H_2O_2 stress on global gene expression. To explore the cellular responses of *B. animalis* subsp. *lactis* strains to oxidative stress we analyzed the transcriptional response of strains BL-04 and DSM 10140 after 5 or 20 min sublethal H_2O_2 exposure. *B. animalis* subsp. *lactis* BL-04 showed a total of 138 significant ($P < 0.05$) differentially expressed (DE) genes after a 5 min exposure, and 27 DE genes after 20 mins (Fig. 5-1). Among the DE genes detected after 5 or 20 mins, 112 (81%) and 22 (82%), respectively, have an assigned function (Fig. 5-1 and Table A-1 in appendix A). In contrast, strain DSM 10140 showed no statistically significant ($P < 0.05$) DE genes at either treatment time compared to control cells.

RT-PCR analysis of 6 selected genes was used to validate microarray data obtained from strain BL-04. As shown in Fig. 5-2, RT-PCR did not detect any contradictions between the two platforms, and there was a strong positive correlation ($r^2 = 0.83$) between the fold-change for gene induction or repression predicted from the microarray and the respective values determined by RT-PCR.

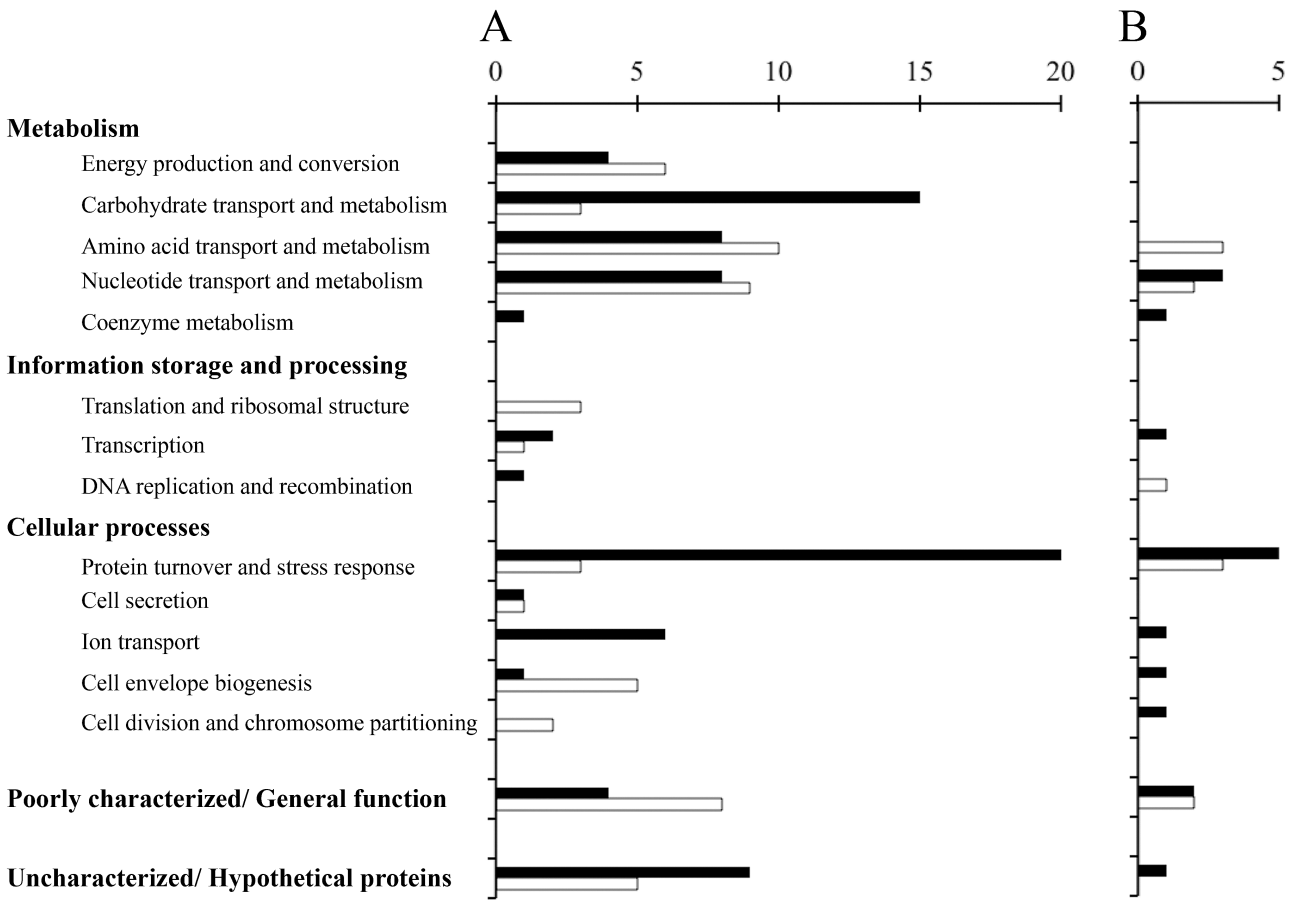


FIG 5-1 Numbers of *B. animalis* subsp. *lactis* BL-04 genes, grouped according to functional category, that were significantly upregulated (black bars) or downregulated (white bars) after 1.25 mM H_2O_2 exposure for 5 min (A) or 20 min (B).

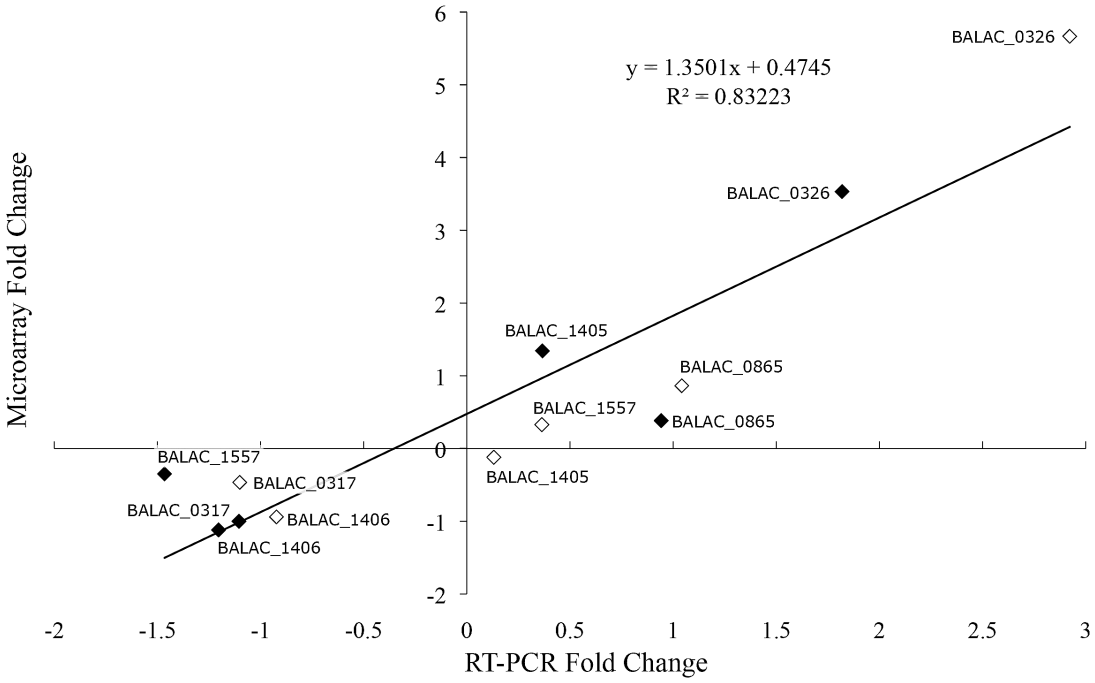


FIG 5-2 Correlation of fold change values from DNA microarray and real-time quantitative PCR results. Fold change values were obtained for the 6 genes listed in Table 1. Symbols denote expression values from *B. animalis* subsp. *lactis* BL-04 cells after 5 min (open diamonds) or 20 min (filled diamonds) exposure to 1.25 mM H_2O_2. The best-fit curve is shown along with the calculated equation and r^2 value.

Bifidobacterium spp. lack the most common genes associated with oxidative stress defense, such as super oxide dismutase and catalase. However grouping of DE genes into predicted functional categories showed that exposure of BL-04 to an oxidative stress triggered up-regulation of genes involved in the thioredoxin reductase system (Table 5-2). Under favorable conditions, this system functions with ribonucleoside reductase to use NADPH to reduce the 2' OH group of ribose for deoxynucleotide production, as well as to maintain cytoplasmic redox for disulfide bond production in proteins (21, 22). During oxidative stress, however, cells can use thioredoxin reductase and peroxiredoxin to direct NADPH toward the removal of oxidative free radicals via the reduction of H_2O_2 and toxic lipid hydroperoxides (23, 24, 25). Schell et al. (26) suggested these enzymes might be one of the primary defense mechanisms against oxidative stress in bifidobacteria, and other research has shown up-regulation of thioredoxin, thioredoxin reductase and peroxiredoxin in response to oxygen stress (27, 28). Interestingly, exposure to bile can also produce an oxidative stress response via generation of oxygen free radicals (29), and Sanchez et al. (30) found bile stress induced a thioredoxin-dependent thiol peroxidase in *B. animalis* subsp. *lactis*. Collectively, our results and these prior data confirm that thioredoxin reductase (Balac_0866) and peroxiredoxin (Balac_0865) provide a primary defense mechanism against oxidative stress in *B. animalis* subsp. *lactis*.

Transcriptome data also showed ribonucleaside-diphosphate reductase alpha and beta chains (Balac_0326 and Balac_0327) were differentially expressed

with a high LFC, as were several other genes (Balac_1501, Balac_1503, Balac_1081, and Balat_0464 [which corresponds to BL-04 gene Balac_0464]) involved in nucleotide turnover (Table 5-2). These genes encode proteins used for dNTP production and to hydrolyze nucleic acids for DNA/RNA turnover and scavenging (31). Under H_2O_2 stress conditions, where peroxiredoxin consumes NADPH for detoxification (and therefore makes it less available for deoxyribonucleotide synthesis), the observed high-level induction of genes for nucleotide turnover could be a reflection of the need to maintain a constant pool of dNTPs to support excision and repair of oxidative-damaged DNA (21, 32, 33).

Additionally, there is an apparent operon in BL-04 (Balac_0440-Balac_0444) that contains genes involved in protein degradation, which showed significant up regulation after 5 min (T1) and a significant down regulation after 20 min (T2) (Table 5-2). Previous research has shown that some bacteria utilize proteolytic enzymes to detoxify proteins that have been irreparably damaged by oxidative stress (34, 35). Our data suggests this operon might be used by BL-04 to perform a similar function.

TABLE 5-2 Differentially regulated genes associated with oxidative stress response of *B. animalis* subsp. *lactis* BL-04.

Gene ID	Predicted function	Log-fold change vs. control	
		T1	T2
Balac_0326	Ribonucleoside-diphosphate reductase beta chain	5.39	2.77
Balac_0327	Ribonucleoside-diphosphate reductase alpha chain	4.23	NS[a]
Balac_0328	NrdI protein / ribonucleotide reductase stimulatory protein	1.71	NS
Balac_0865	Peroxiredoxin	1.10	NS
Balac_0866	Thioredoxin reductase	1.84	1.19
Balac_0118	Oxidoreductase	1.40	NS
Balac_0120	Vanillate O-demethylase oxidoreductase / ferric reductase	1.59	NS
Balac_0121	Flavodoxin	2.14	NS
Balac_0123	Flavodoxin	1.54	NS
Balac_1314	Anaerobic ribonucleoside-triphosphate reductase activating protein	1.05	NS
Balac_1315	Anaerobic ribonucleoside-triphosphate reductase	0.72	NS
Balac_0573	NTP pyrophosphohydrolases including oxidative damage repair enzymes	NS	1.34
Balac_0025	Oxidoreductase	0.56	NS
Balac_1337	MoxR-like ATPase	-0.88	NS
Balac_0086	Penicillin-binding protein	-0.74	NS
Balac_1247	DNA repair protein recO	-0.97	NS
Balac_1114	RecA protein	0.58	NS
Balac_1212	LexA repressor	0.60	NS
Balac_1437	Multidrug resistance protein B	0.52	-1.22

Balac_1555	DnaJ-class molecular chaperone	NS	-1.34
Balac_1556	GrpE protein	0.47	NS
Balac_0440	Acyl-coenzyme A:6-aminopenicillanic-acid-acyltransferase precursor	NS	-1.63
Balac_0441	Aminopeptidase C	0.76	NS
Balac_0442	Glutamate/gamma-aminobutyrate antiporter	0.74	-1.37
Balac_0443	Carboxypeptidase S1	1.28	-1.27
Balac_0444	Amino acid permease	0.77	NS
Balac_1501	Sugar kinases, ribokinase family	3.03	2.41
Balac_1502	tetracycline resistance permease/tetracycline efflux pump/MFS[b] transporter/	2.98	2.53
Balac_1503	Inosine-uridine preferring nucleoside hydrolase	2.79	2.54
Balat_0464	5'-nucleotidase	1.05	NS
Balac_1081	Ribonuclease D	1.25	NS
Balac_1597	Raffinose transport system permease protein	3.46	NS
Balac_1598	Raffinose transport system permease protein	3.20	NS
Balac_1599	Raffinose-binding protein	3.12	NS

[a]NS, Not Significant
[b]MFS, Mutli-facilitator superfamily

Finally, 28 genes were associated with energy production or sugar transport in strain BL-04, with 19 (68%) of those genes being upregulated in response to H_2O_2 stress (Fig. 5-1). In this study, the DE genes involved in sugar metabolism with the highest LFC included those involved in raffinose (Balac_1597 to Balac_1601) and maltose transport and metabolism (Balac_1567 to Balac_ 1573) (see Table S1 in appendix B). The influence of these sugars on H_2O_2 resistance was not explored here, but other studies have suggested that complex carbohydrates can enhance bile salt resistance in bifidobacteria (36, 37).

Membrane fatty acid analysis. Several studies have shown cell envelope lipid composition plays a crucial role in bacterial response to environmental stresses (38-42). We therefore investigated the membrane fatty acid composition of BL-04 and DSM 10140 after H_2O_2 exposure. Analysis showed no significant change in membrane composition in BL-04 cells grown in media containing 2.55 mM H_2O_2 for 5 min (T1) or 60 min (T2) compared to cells grown in control media (Fig. 5-3). In contrast, strain DSM 10140 showed a significant decrease ($P<0.05$) in $C_{16:0}$ and a significant increase ($P<0.05$) in $C_{18:1n9}$ after exposure to 2.55 mM H_2O_2 (Fig 5-3). Surprisingly, direct comparison between the bifidobacteria strains showed dramatic differences even in control cells, with BL-04 having 20% more $C_{16:0}$, and 15-20% lower amount of $C_{18:1n9}$. Both strains had similar total amounts of $C_{16:1}$ with BL-04 having predominantly $C_{16:1n7}$ and DSM 10140 having more $C_{16:1n9}$ (Fig. 5-3). These differences are similar to those seen in other bacteria (43, 44, 45) and likely factor

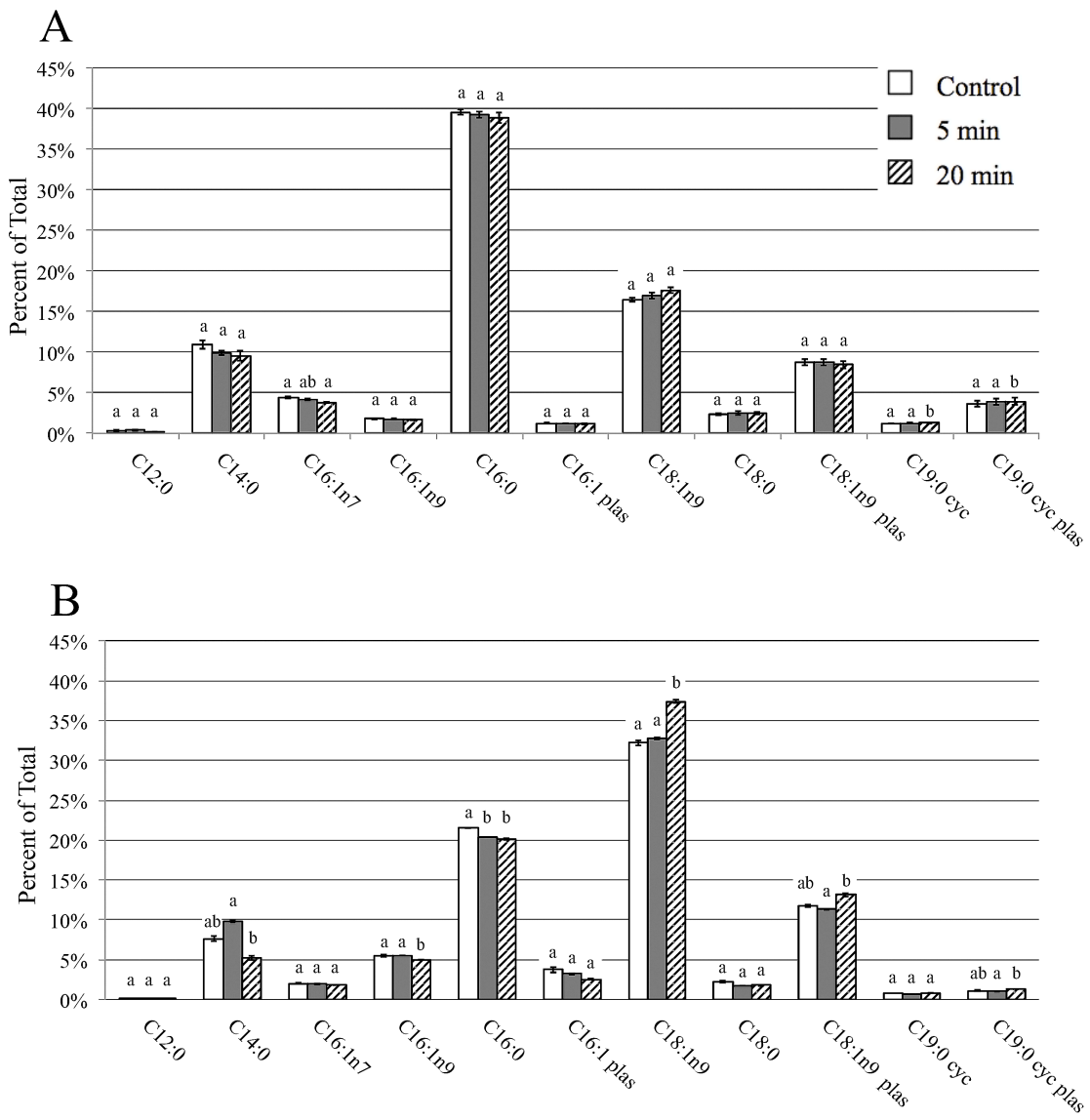


FIG 5-3 Membrane fatty acid composition for *B. animalis* subsp. *lactis* BL-04 (A) and DSM 10140 (B). The graphs shows data from cells grown in MP5 media with no H_2O_2 (control) and cells exposed to 1.25 mM H_2O_2 in MP5 broth for 5 or 20 min. Error bars correspond to the standard error of the mean (SEM). Means with the same letters within each strain are not significantly different ($P < 0.05$).

into the increased survival of BL-04 under oxidative stress compared to DSM 10140 (14).

In an effort to identify the basis for the dramatic differences we observed in the gene expression profiles and membrane lipid composition of BL-04 versus DSM 10140, we reviewed the comparative genome analysis of these bacteria (12). The two strains are highly clonal with only 39 coding single nucleotide polymorphisms and 4 insertion/deletions totaling 443 base pairs. However, we found one of these lesions produced a 45-bp deletion in a BL-04 gene (Balac_0771) predicted to encode a long chain fatty acid-coenzyme A (CoA) ligase. In other bacteria, this gene has been shown to activate exogenous long chain fatty acids for incorporation into the cellular membrane (46), and therefore might result in a different membrane lipid profile for MP5-grown BL-04 compared to DSM 10140. To test whether this lesion affected the ability of BL-04 to incorporate exogenous fatty acids into its membrane, both strains were grown in MP5 medium modified to contain 1% Tween 80 ($C_{18:1}$), 1% Tween 20 ($C_{12:0}$), or no fatty acids, then lipids were extracted for membrane fatty acid analysis.

As shown in Fig. 5-4, DSM 10140 cells grown in MP5 with Tween 20 showed dramatic and significantly higher ($P < 0.05$) amounts of $C_{12:0}$ and significantly decreased ($P < 0.05$) the pooled total of $C_{18:1n9}$ and its derivatives ($C_{19:0}$ cyclic propanol [Cyc], $C_{19:0}$ cyc plasmologens [Plas] and $C_{18:1n9}$ Plas) (19) than cells grown in MP5 with no fatty acid supplementation (21.4% versus 35.7%, respectively). Conversely, when DSM 10140 cells were grown in MP5 containing Tween 80, their

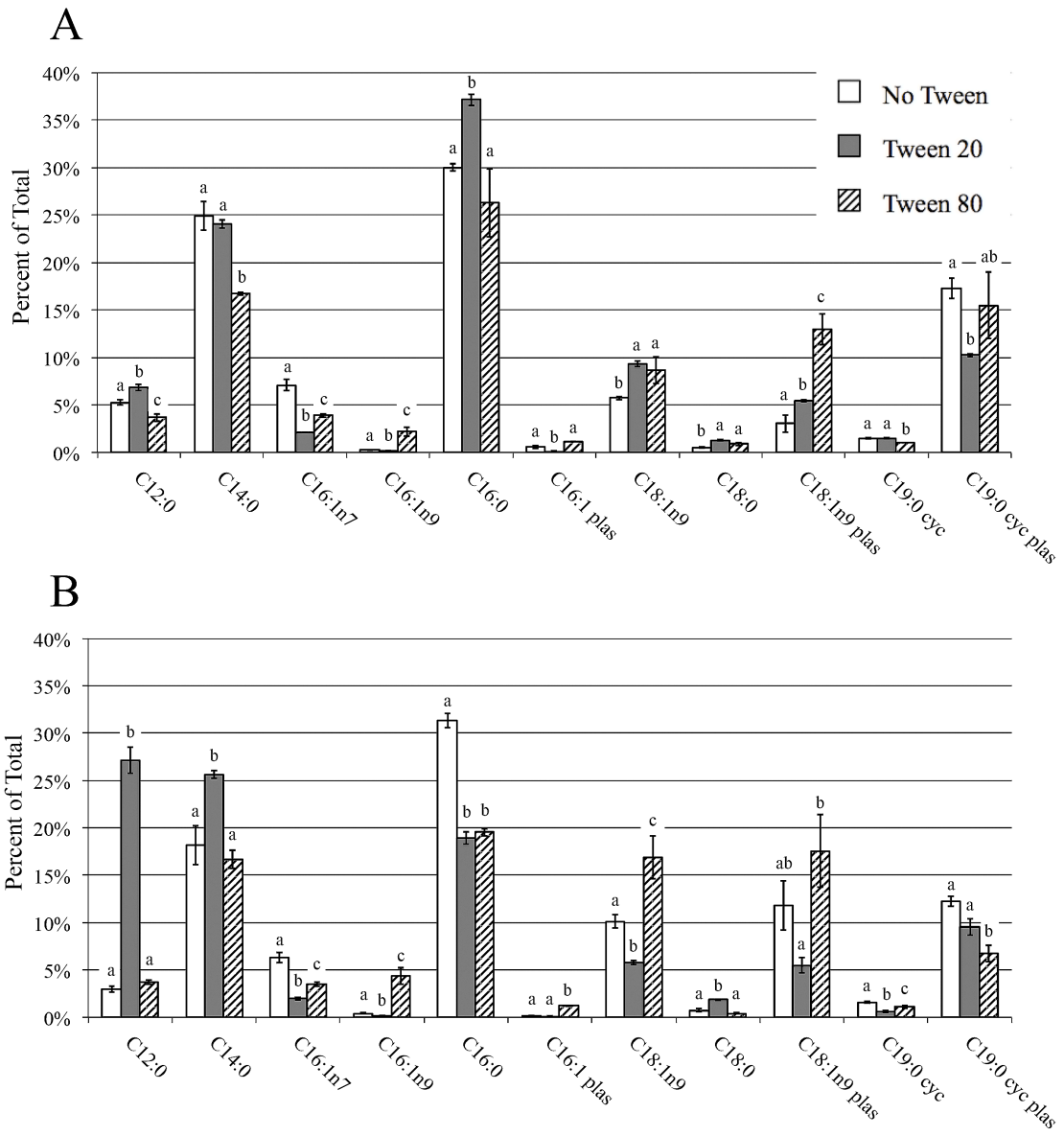


FIG 5-4 Membrane fatty acid composition as a function of growth medium composition for *B. animalis* subsp. *lactis* BL-04 (A) and DSM 10140 (B) cells. The graphs shows data from cells grown in MP5 media with no exogenous fatty acids and cells grown in MP5 with 1% Tween 20 ($C_{12:0}$) or 1% Tween 80 ($C_{18:1\ n9}$). Error bars correspond to the standard error of the mean (SEM). Means with the same letters within each strain are not significantly different ($P < 0.05$).

membrane showed significantly ($P < 0.05$) higher percentages of $C_{18:1n9}$, as well as an increase in $C_{18:1n9}$ Plas which was not significant, versus cells grown in MP5 without added fatty acid (Fig. 5-4). DSM 10140 cells grown in MP5 supplemented with Tween 20 or 80 also had significantly less ($P < 0.05$) $C_{16:0}$ than cells grown in MP5 without fatty acids. These results confirm DSM 10140 is able to efficiently incorporate exogenous fatty acids into its lipid membrane.

In contrast to DSM 10140, membrane lipid profiles of BL-04 cells grown under the same conditions showed far less change in response to the exogenous fatty acid type, which supports our hypothesis that the 45-bp lesion in Balac_0771 impairs the function of its cognate enzyme. Supplementation with Tween 20, for example, did produce a significant increase ($P < 0.05$) in the membrane level of $C_{12:0}$ relative to cells grown without added fatty acids, but the degree of change was substantially lower than seen in DSM 10140 (from 5.2 to 6.8% in BL-04 versus 3.0 to 27.3% in DSM 10140). Additionally, levels of $C_{18:1n9}$ and its derivatives were not significantly different ($P > 0.05$) from that found in BL-04 cells grown without fatty acid supplementation (totals of 26.5% versus 27.5%, respectively) (Fig. 5-4). Growth of BL-04 in MP5 with Tween 80 did produce a significant increase ($P < 0.05$) in the concentration of $C_{18:1n9}$ and its derivative fatty acids relative to cells grown in MP5 without fatty acids (totals of 38.1% versus 27.5%, respectively). These differences, which would have been present in stress-treated cells, could affect membrane fluidity and, potentially, transduction of environmental stress signals, either of which could explain the observed contrasts in intrinsic and inducible H_2O_2

stress resistance (14). As a whole, these data support our hypothesis that the mutation in the BL-04 long chain fatty acid-CoA ligase limits the ability of this strain to incorporate certain exogenous fatty acids into its cytoplasmic membrane.

In our experiments with different exogenous fatty acids, we noted that DSM 10140 cells grown in MP5 containing no fatty acids had a gross lipid profile that most closely matched the lipid profile of BL-04 grown in media containing Tween 80 (Table 5-3). Because membrane fluidity could influence the efficiency of environmental stress triggers, we wondered if an inducible stress response in DSM 10140 might be restored by modification of its membrane fatty acid composition to more closely match the profile of BL-04. To explore this possibility, DSM 10140 cells were grown in MP5 media that contained no supplemented fatty acids, treated with a sublethal H_2O_2 concentration [1.25 mM], and subsequently exposed to a lethal H_2O_2 concentration [2.55 or 5.25 mM] (14). Results showed no significant change in survival after H_2O_2 challenge of induced versus control cells grown in MP5 with Tween 80, and a significant decrease in survival of induced cells compared to control cells grown in media with no exogenous fatty acids (Fig. 5-5). As is also shown in Fig. 5-5, however, cells grown with no exogenous fatty acid had significantly greater survival ($P < 0.05$) after lethal challenge at 5.25 mM H_2O_2 as compared to cells grown in MP5 with Tween 80 (Fig. 5-5). The increase in intrinsic H_2O_2 resistance could be associated with the higher percentage of cyclic fatty acids in the membrane (47, 48).

TABLE 5-3 Membrane fatty acid (FA) composition of *B. animalis* subsp. *lactis* strains grown with different exogenous FA.

	Mean % of each FA species in total cytoplasmic membrane lipid pool				
Strain and treatment	Cyclic	Plasmalogen[c]	Saturated[d]	Unsaturated[e]	Saturated/unsaturated
Bl-04					
No Tween	18.81	22.03	62.35	37.65	1.66
Tween 20[a]	11.71	16.04	70.07	29.93	2.34
Tween 80[b]	16.61	30.52	49.87	50.13	0.99
DSM 10140					
No Tween	13.87	25.08	54.94	45.06	1.22
Tween 20[a]	10.51	15.47	74.41	25.59	2.91
Tween 80[b]	7.96	26.60	43.39	56.61	0.77

[a]Polyoxyethylene (20) sorbitan monolaurate ($C_{12:0}$)

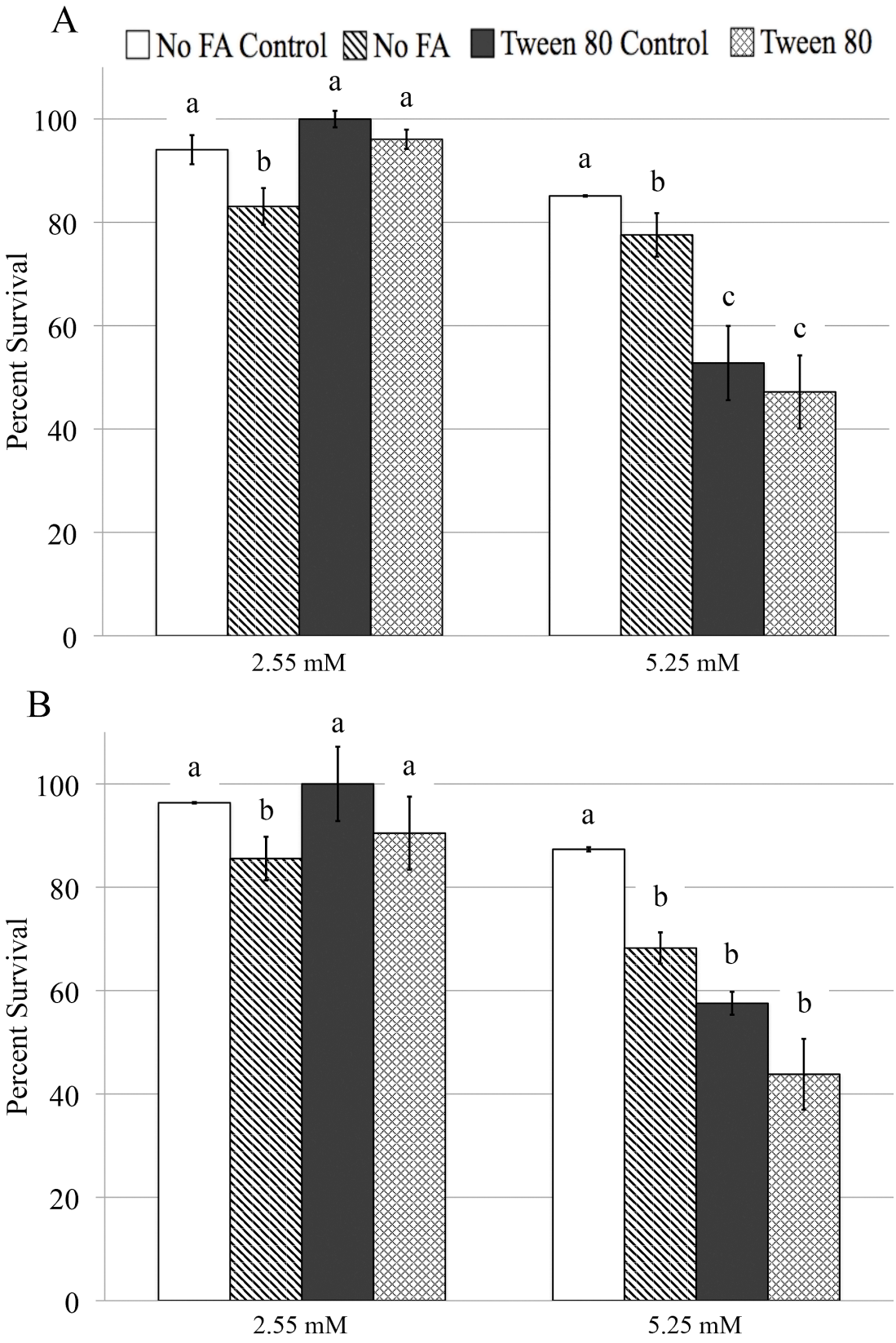
[b]Polyoxyethylene (20) sorbitan monooleate ($C_{18:1}$)

[c]Plasmologens (ether-linked lipids)

[d]Percentage of saturated FA in membrane including cyclic FA and plasmologens

[e]Percentage of unsaturated FA in the membrane including plasmologens

FIG 5-5 Experimental stress induction in *B. animalis* subsp. *lactis* DSM 10140. Graph shows percent survival of DSM 10140 cells after 20 min (A) or 60 min (B) exposure to a sublethal (1.25 mM) H_2O_2 followed by 30 min challenge at lethal concentrations of 2.55 mM H_2O_2 or 5.25 mM H_2O_2. White bar, cells grown in MP5 with no exogenous FA source and no induction (control); hashed bar, cells grown with no exogenous FA source and given induction treatment; filled bar, cells grown in MP5 with Tween 80 as FA source and no induction (control); cross-hatched bar, cells grown in MP5 with Tween 80 and given induction treatment. Each value is the mean of four replicates. Error bars correspond to the standard error of the mean (SEM). Means with the same letters within each strain are not significantly different ($P < 0.05$).


A
No FA Control
No FA
Tween 80 Control
Tween 80
Percent Survival
100
80
60
40
20
0
a
b
a
a
a
b
c
c
2.55 mM
5.25 mM
B
Percent Survival
100
80
60
40
20
0
a
b
a
a
a
b
b
b
2.55 mM
5.25 mM

Previous studies have shown that in response to low pH and osmotic stress, cells modify their membrane through chain length, saturation and cyclopropanation of fatty acids, which alters the transition temperature of the membrane and makes it less permeable to organic acids and salts (44, 45, 49). More importantly, cyclopropanation decreases the susceptibility of the cell membrane to lipid peroxidation by stabilizing the unsaturated bond by addition of a methyl group (50). These properties would make the membrane less permeable to oxidative free radicals and more resistant to lipid peroxidation.

In summary, *B. animalis* subsp. *lactis* BL-04 and DSM 10140 are highly clonal yet display significant differences in their intrinsic and inducible resistance to H_2O_2 (14). Transcriptome data demonstrate H_2O_2 exposure triggers induction of an oxidative stress response in BL-04, but this mechanism is somehow impaired in DSM 10140. Genetic and membrane lipid data suggest some of the differences in H_2O_2 resistance between these cells may be associated with membrane lipid composition, which in turn is affected by the activity of a long chain fatty acyl-CoA ligase which is functional in DSM 10140 but impaired in BL-04. However, confirmation of this relationship will require functional studies involving genetic manipulation of *B. animalis* subsp. *lactis*, where genetic tools are only poorly developed. While efforts to restore an inducible H_2O_2 stress response in DSM 10140 via modification of its CMFA composition were unsuccessful, modification did significantly increase intrinsic H_2O_2 resistance. These data show deliberate H_2O_2

stress induction or membrane lipid modification can be used to significantly improve H_2O_2 resistance in *B. animalis* subsp. *lactis*.

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CHAPTER 6

TRANSCRIPTOME ANALYSIS OF *BIFIDOBACTERIUM LONGUM* STRAINS THAT SHOW A DIFFERENTIAL RESPONSE TO HYDROGEN PEROXIDE STRESS

ABSTRACT

Consumer and commercial interest in foods containing probiotic bifidobacteria is increasing worldwide. However, because bifidobacteria are anaerobic, oxidative stress can diminish cell viability during production and storage of bioactive foods. We previously found *Bifidobacterium longum* strain NCC2705 had significantly greater intrinsic and inducible resistance to hydrogen peroxide (H_2O_2) than strain D2957. Here, we explored the basis for these differences by examining the transcriptional responses of both strains to sub-lethal H_2O_2 exposure for 5 or 60 min showed NCC2705 had 288 genes that were differentially expressed after the 5-min treatment and 114 differentially expressed genes after the 60-min treatment. In contrast, strain D2957 had only 21 and 90 differentially expressed genes after the 5- and 60-min treatments, respectively. Both strains showed up-regulation of genes coding enzymes implicated in oxidative stress resistance, such as thioredoxin, thioredoxin reductase, peroxiredoxin, ferredoxin, glutaredoxin, and anaerobic ribonucleotide reductase, but induction levels were typically highest in NCC2705. Compared to D2957, NCC2705 also had more up-regulated genes involved in transcriptional regulation and more down-regulated genes involved in sugar

transport and metabolism. These results provide a greater understanding of the molecular basis for strain variability in oxidative stress resistance of *B. longum* and reveal possible methods to promote their survival in bioactive food products.

INTRODUCTION

Consumption of food or food ingredients with bioactive properties has increased in recent years and probiotic bacteria represent one of the most promising categories of bioactive food ingredients. The term probiotic refers to living microorganisms which, when ingested at sufficient numbers, exert a beneficial effect on the host organism beyond inherent general nutrition (1). Currently, bacteria from the genera *Lactobacillus* and *Bifidobacterium* are the most widely used probiotic bacteria added functional foods. Bifidobacteria are Gram-positive, non-acid-fast, non-spore forming, non-motile, anaerobic, catalase negative rods of irregular shape, with a G+C content of 55-67%, and are part of the normal gastrointestinal flora in human adults (2, 3). Bifidobacteria are thought to promote or provide several health related functions, including a decrease in severity of the side effects associated with antibiotics use, a reduced incidence of infection in patients receiving irradiation therapy, a decrease in the duration of diarrhea due to various etiologies, improved lactose digestion, a reduced frequency of allergic reactions, normalization of blood lipid composition, and a decrease in gut transit time (4-8). Although no conclusive data is available on a minimal effective dose of probiotics in humans, results from several clinical trials suggest a direct dose-effect

correlation (9, 10, 11). In practice this means that bifidobacteria need to be delivered at very high concentrations in bioactive foods to function as a probiotic. At present, yogurt or fermented milks are the most common vehicle foods for delivery of probiotic bifidobacteria, but cheese, ice cream, infant formula, fruit juice, and other foods are also used to a lesser extent (12). One of the major hurdles to production and storage of bioactive foods containing bifidobacteria as a probiotic is oxidative stress. This is because bifidobacteria are anaerobic and lack common enzymes such as superoxide dismutase that detoxify oxidative free radicals in the presence of oxygen (13, 14, 15). We previously found *Bifidobacterium longum* strain NCC2705 had greater intrinsic and inducible resistance to H_2O_2 than strain D2957. Specifically, 60 min exposure to sublethal concentration (1.25 mM) of H_2O_2 was shown to significantly improve survival ($P < 0.05$) of *B. longum* NCC2705 at lethal (5.25mM) H_2O_2 concentrations, but a similar response was not detected with strain D2957 (16). Additionally, *B. longum* NCC2705 showed a 1.5-fold higher intrinsic H_2O_2 resistance than D2957 (16). The purpose of this study was to explore the physiological basis for these differences by determining the transcriptional responses of *B. longum* NCC2705 and D2957 to sublethal H_2O_2 exposure.

MATERIALS AND METHODS

Culture conditions. Bacterial strains were maintained as glycerol freezer stocks at -80°C, and working cultures were prepared by two successive transfers (1% innoculum [vol/vol]) into peptonized milk medium (MP5) (16) with anaerobic

incubation at 37°C for 18 h. Batch cultures of each strain were prepared by dilution of the working culture to an absorbance at 600 nm (A_{600}) of 1.0 in MP5 medium, then inoculated at 1% (vol/vol) into 1 L of MP5 in a New Brunswick BioFlo III fermenter (New Brunswik Scientific, Edison, New Jersey). Cells were incubated at 37°C with an agitation rate of 100 rpm to prevent sedimentation. A gas mixture of 5% CO_2 and 95% N_2 was continuously passed over the headspace of the fermenter to achieve anaerobic conditions, and the pH was maintained at 6.5 by automatic addition of 15% (vol/vol) NH_4OH. Bifidobacteria were incubated until the cells reached early stationary-phase (approximately 12 h) (16).

RNA isolation. RNA isolation was performed as previously described (17). Cells were grown in batch culture under pH control to early stationary phase and then samples (5 mL) were harvested by centrifugation at 7500 x *g* for 10 min. The cell pellets were suspended in 50 mL of pre-warmed MP5 media containing a sublethal H_2O_2 concentration of 1.25mM (16) for 5 (T1) or 60 (T2) min. Immediately after treatment, 100mL of RNAprotect bacterial reagent (Qiagen, Inc., Valencia, CA) was added to the cell suspensions to stop transcription and prevent mRNA degradation. A control sample was also prepared which was not exposed to H_2O_2. Cells in RNAprotect were held at room temperature for 10 min, then collected by centrifugation at 9500 x *g* for 10 min and stored at -20°C until RNA isolation.

Cell pellets were thawed at room temperature and suspended in 900 µL of a lysis solution containing 20 mg lysozyme (Sigma-Aldrich) and 20 U of mutanolysin (Sigma-Aldrich) per mL in 1mM TE buffer adjusted to pH 7.6. Samples were

incubated for 30 min at 37°C on a shaker incubator at 240 rpm, after which 20 µl of proteinase K (Omega Bio-Tek Inc., Norcross, GA) (>600 mAU/ml) was added and the samples were returned to the shaker/incubator for 30 min. The RNA was then isolated using the Aurum total RNA mini kit (Biorad, Hurcules, CA) following the vendor's recommended procedures. The quantity of recovered RNA was measured with a NanoDrop 8000 spectrophotometer (ThermoFisher Scientific, Waltham, MA) and the quality of the RNA was assayed using an Agilent 2100 bioanalyzer (Agilent Technologies, Inc., Waldbronn, Germany). Samples that had sufficient quantities (>10µg) of quality RNA were stored at -80°C until needed.

Synthesis and labeling of cDNA. cDNA synthesis and labeling was performed as described previously (17). cDNA was synthesized and labeled as recommended by the Affymetrix (Santa Clara, CA) protocol for prokaryotic target preparation in the GeneChip Expression Analysis Technical Manual (media.affymetrix.com). The cDNA was fragmented into approximately 50-100 bp using DNAse I and labeled with GeneChip DNA labeling reagent (Affymetrix, Santa Clara, CA) and terminal deoxynucleotidyl transferase (Promega, Madison, WI). Fragmentation labeling efficiency was measured by gel shift assay.

DNA microarrays. DNA microarray hybridization and statistical analysis was performed as described previously (17). Sample hybridization was performed at the Center for Integrated Bio-systems at Utah State University against a custom Affymetrix bifidobacterial DNA microarray designed to include 3113 shared plus unique chromosomal genes predicted to occur in *B. longum* NCC2705 and D2957.

The only predicted coding sequences not included in the microarray design were redundant transposases and rRNA genes. Hybridization was performed according to the Affymetrix protocol for prokaryotic target hybridization in the GeneChip Expression Analysis Technical Manual using a hybridization temperature of 50°C. The DNA microarrays were scanned using the HP GeneArray scanner (Affymetrix, Santa Clara, CA) to generate raw intensity values for each probe.

Statistical analysis of microarray data was performed using Biocunductor (www.bioconductor.org) in the open source statistical platform R (www.r-project.org). The raw probe data was preprocessed using the RMA-MS method (18) and filtered to only include genes that had a high signal intensity and a low coefficient of variation. To test for differential expression, the preprocessed, filtered data was analyzed using the limma/eBayes method (19). Genes were determined to be significantly differentially expressed if they had a false discovery rate corrected P-value < 0.05. The significantly differentially expressed genes were grouped according to function and by treatment times and strain.

Microarray validation. To validate the microarray data, quantitative real-time PCR (RT-PRC) was performed as described by Smeianov et al. (20) for 6 different genes (Table 6-1) using cDNA produced after each treatment. A log-fold change (LFC) was calculated between control and treatment samples, and graphed vs. the LFC calculated from the microarray data.

Whole genome sequencing of *B. longum* D2957. Total genomic DNA was isolated from strain D2957 using the Masterpure Gram Positive DNA purification kit

Table 6-1 Target genes oligonucleotide primers for RT-PCR.

Protein function (Gene ID)	Primer Sequence		Amplicon size (bp)	Annealing temp (°C)
	Forward	Reverse		
Peroxiredoxin (BL0615)	GTGGCTGAGCGTGGCGACTT	ACCTGGTCGCCGTGCTCGTA	146	61.5
Glutaredoxin (BL0668)	CACCAAGCGCCAGCTCACCA	GCGTGATGACCACCGGAGCC	122	61.5
Thioredoxin (RBLN00690) (BLD_0988)	GCGCGTTCGGCCCGATTTTC	GGGCCAGATCCTGGTTGGCG	99	61.5
Ferredoxin (BL01563)	TACGAGGGTTCCCGCTCGCT	AAGATGGCCTCGGTGGGGCA	89	61.5
Ferridoxin (BL1725)	GGCTACGCGGGTGCATTGGT	CCGGGGTGAAACGTGGGTCG	105	61.5
Anaerobic Ribonucleotide Reductase (BL1752)	TCAAGGGGCGTTACACCGGC	GGCGCGAGCCACATCGTACA	97	61.5
dnaK (BL0520)	CCCAGCGTCAGGCCACCAAG	GCTGCGGTCGGCTCGTTGAT	79	61.5

(Epicenter Biotechnologies, Madison WI) then high-throughput whole-genome shotgun DNA sequencing and assembly was performed at the Utah State University Center for Integrated Biosystems using the Roche 454 GS Titanium pyrosequencer platform. The sequence data was assembled into a 2.33 Mbp draft genome consisting of 13 contigs, and automated genome annotation was performed using the RAST algorithm (rast.nmpdr.org).

Membrane fatty acid analysis. To determine whether H_2O_2 exposure altered cytoplasmic membrane fatty acid (CMFA) composition, cells were grown in batch culture as described, and treated with a sublethal H_2O_2 concentration of 1.25mM for 5 (T1) or 60 (T2) min. Twenty mL samples were collected by centrifugation at 5000 × *g* for 5 min and then washed twice with phosphate buffered saline. Membrane fatty acids were isolated from the pelleted cells according to the protocol of Sasser (21) and identified using gas chromatography as described previously (22). An untreated control sample was also prepared. Amounts of individual fatty acids were calculated as a percent of total and a two-tailed student *t* test was used to determine differences in means between samples.

Microarray data accession number. Microarray hybridization data have been deposited in the Gene Expression Omnibus under accession number GSE44709.

WGS sequence accession number. The Whole Genome Shotgun sequence for *B. longum* D2957 has been deposited at DDBJ/EMBL/GenBank under the

accession AQGL00000000. The version described in this paper is the first version, AQGL01000000.

RESULTS AND DISCUSSION

Influence of H_2O_2 stress on global gene expression. To explore possible causes for differences in H_2O_2 resistance between *B. longum* NCC2705 and D2957, we analyzed their transcriptional changes after 5 or 60 min sublethal H_2O_2 exposure. Because our original DNA microarray design was based on the two *B. longum* genomes (NCC2705 and DJO10A) that were publicly available at the time, whole genome pyrosequencing of *B. longum* D2957 was first performed to better interpret the transcriptome results for this strain and to identify genes that were unlikely to hybridize to the microarray. Prior data from whole genome hybridizations with *Bifidobacterium* strains against the custom microarray (unpublished data) was used to estimate the cutoff threshold for hybridization of D2957 genes to the array. Those results suggested D2957 genes encoding proteins with ≤ 77% amino acid identity to array targets were unlikely to hybridize, and therefore deemed not detectable. Among the 2,073 predicted open reading frames in the D2957 genome, 495 were below the cutoff value (see supplementary data Table A-2 in appendix A), and 350 (71%) of these genes encoded hypothetical proteins or had only general function.

Among the 1,578 *B. longum* D2957 genes that were represented on the array, 21 showed statistically significant ($P < 0.05$) differentially expressed (DE) genes

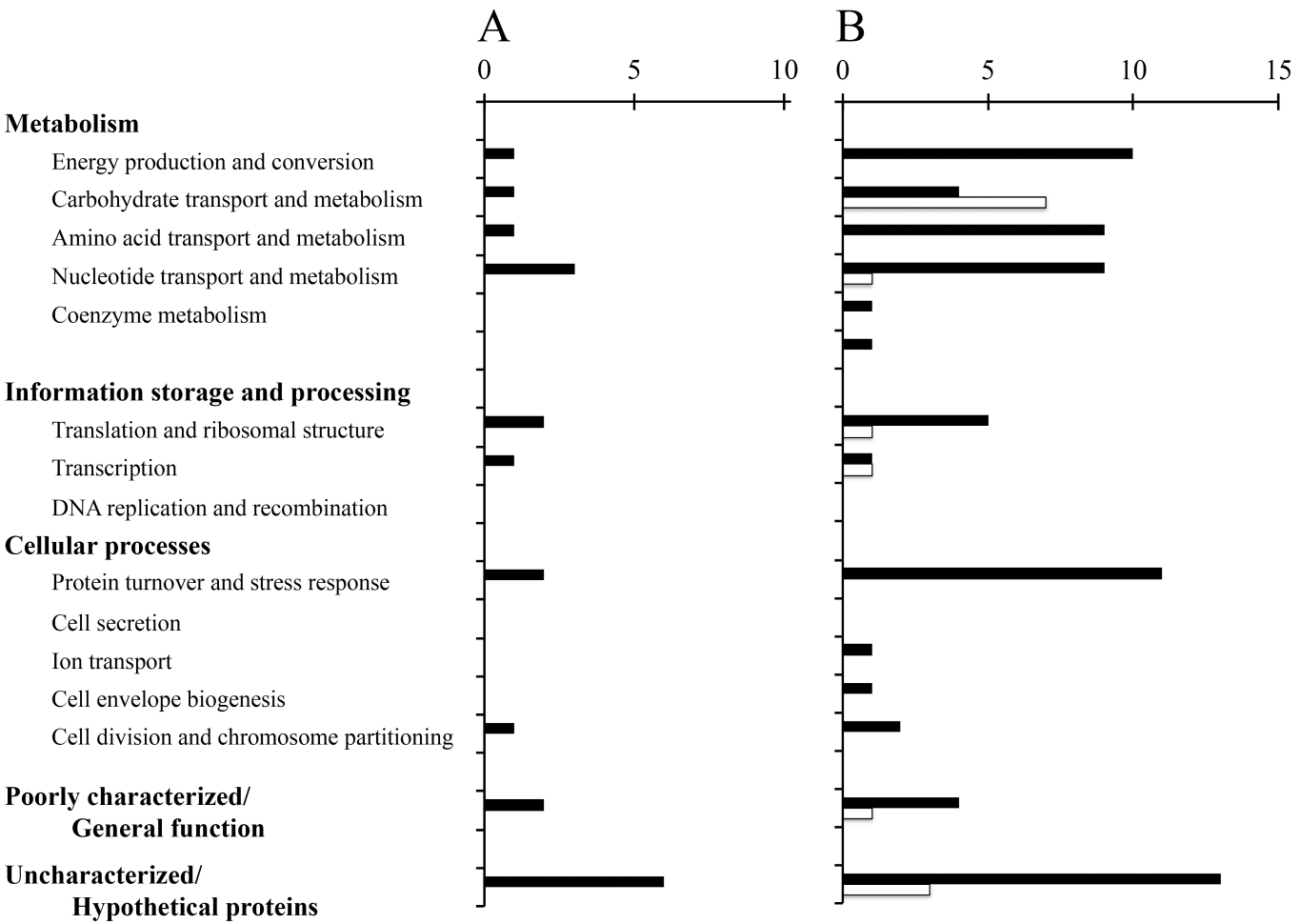

FIG 6-1 Numbers of *B. longum* D2957 genes, grouped according to functional category, that were significantly upregulated (black bars) or downregulated (white bars) after 1.25 mM H_2O_2 exposure for 5 min (A) or 60 min (B)

compared to control cells after 5 min exposure to a sublethal H_2O_2 exposure, and 90 DE genes were detected after 60 min exposure. Of these, 12 (60%) and 65 (76%) of the genes that were DE after 5 and 60 mins, respectively, have an assigned function (Fig. 6-1 and Table A-2).

In contrast, *B. longum* NCC2705 showed a total of 288 significantly ($P < 0.05$) DE genes after a 5 min exposure, and 114 DE genes after 60 mins (Fig. 6-1). Among the DE genes detected after 5 or 60 mins, 192 (69%) and 83 (74%), respectively, have an assigned function (Fig. 6-2 and Table A-2 in appendix A).

RT-PCR analysis of 6 selected genes was used to validate microarray data obtained from *B. longum* NCC2705 and D2957. RT-PCR did not detect any contradictions between the two platforms (Fig. A-1 in appendix A), and there was a positive correlation ($r^2 = 0.68$ and 0.63 respectively) between the fold-change for gene induction or repression predicted from the microarray and the respective values determined by RT-PCR.

Due to their anaerobic nature, *Bifidobacterium* ssp. lack the most common enzymes associated with oxidative stress defense, such as super oxide dismutase and catalase. However, other anaerobic bacteria have developed different systems to mitigate the toxic effects of H_2O_2 such as NADH peroxidases, peroxiredoxin, ferritine-like iron binding proteins and DNA repair enzymes (23, 24). Initial grouping of DE genes into predicted functional categories showed H_2O_2 exposure triggered up-regulation of genes involved in several such systems in both strains

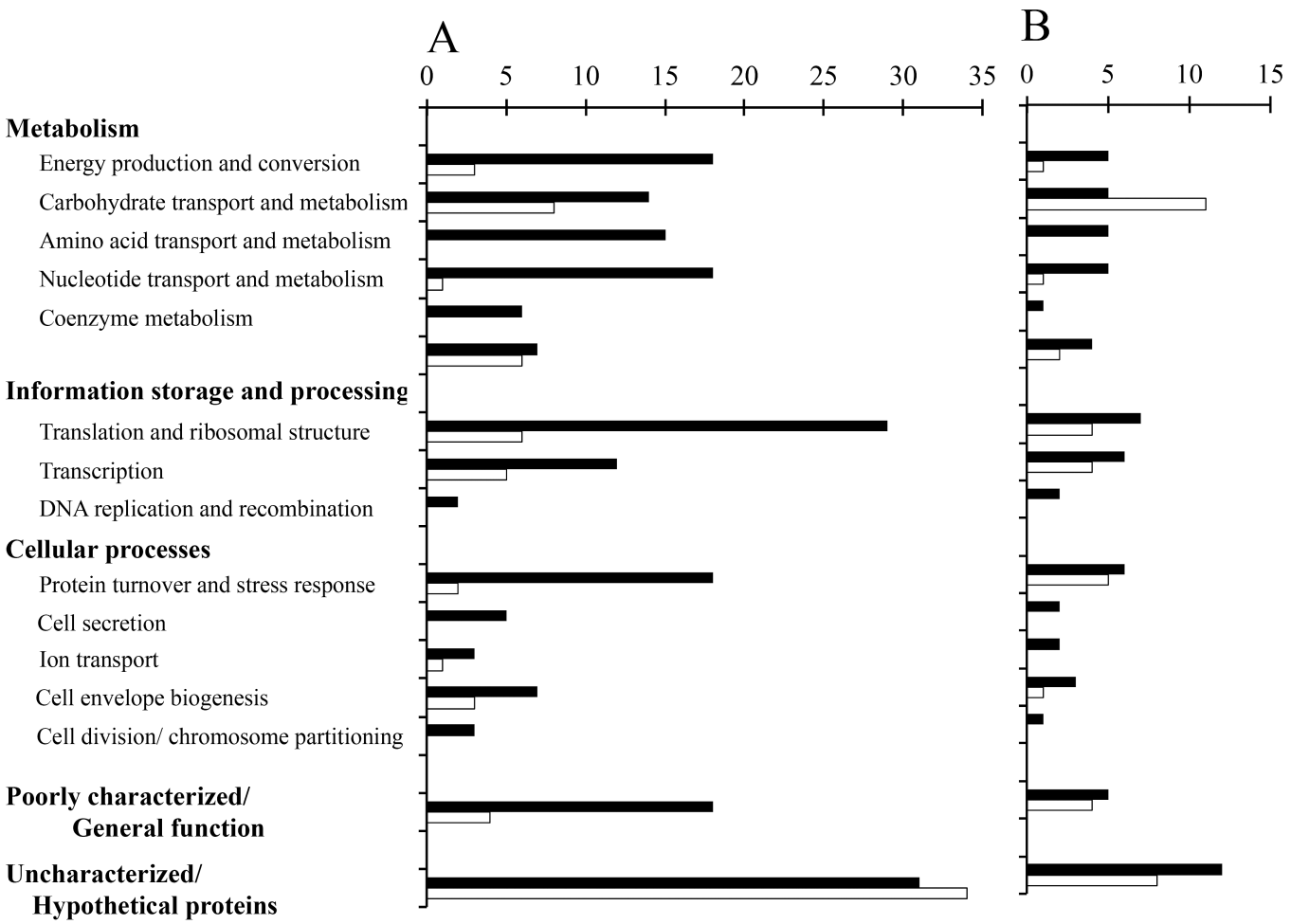

FIG 6-2 Numbers of *B. longum* NCC2705 genes, grouped according to functional category, that were significantly upregulated (black bars) or downregulated (white bars) after 1.25 mM H_2O_2 exposure for 5 min (A) or 60 min (B).

Table 6-2 Examples of differentially regulated genes associated with oxidative stress response of *B. longum* strains.

General functional category and gene ID	Predicted Function	Fold Change vs. Control			
		NCC2705		D2957	
		T1	T2	T1	T2
BL0555	Trypsin-like serine proteases, typically periplasmic,	1.68	NS[a]	NS	NS
BL0781	Putative intracellular protease/amidase	0.94	0.96	NS	NS
BL0944	ATP-dependent endopeptidase clp proteolytic subunit clpP	NS	NS	NS	0.96
BL0945	Protease subunit of ATP-dependent Clp proteases	0.79	NS	NS	1.48
BL1682	ATP-dependent Zn proteases	0.99	NS	NS	NS
BL0139	NAD(P)H-dependent FMN reductase	NS	NS	NS	1.92
BL0399	Protoporphyrinogen oxidase	1.06	NS	NS	NS
BL0552	NADPH-dependent glutamate synthase beta chain and related oxidoreductases	NS	1.03	NS	NS
BL0614	Thioredoxin reductase	1.92	1.56	NS	0.88
BL0615	Peroxiredoxin	1.36	1.29	NS	1.16
BL0668	Glutaredoxin and related proteins	1.79	1.59	1.57	1.49
BL0669	NrdI protein/ ribonucleotide reductase stimulatory protein	1.42	NS	NS	NS

BL0670	Ribonucleotide reductase, alpha subunit	0.92	NS	NS	NS
BL0671	Ribonucleoside-diphosphate reductase beta chain	1.40	NS	NS	0.73
BL1563	Ferredoxin	0.86	NS	NS	NS
BL1750	Exodeoxyribonuclease VII small subunit	0.75	1.10	NS	NS
BL1752	Oxygen-sensitive ribonucleoside-triphosphate reductase	1.88	1.59	NS	NS
BL1753	Anaerobic ribonucleoside-triphosphate reductase activating protein	0.95	0.89	NS	NS
BLD_0988	Thioredoxin	NS	NS	NS	1.52
BL0001	Cold shock proteins	0.75	NS	NS	0.85
BL0002	60 kDa chaperonin GROEL	NS	NS	NS	1.09
BL0010	ATPases with chaperone activity, ATP-binding subunit	0.89	NS	NS	NS
BL0355	Predicted nuclease of the RecB family	-0.82	NS	NS	NS
BL0517	Molecular chaperone (small heat shock protein)	NS	0.92	NS	NS
BL0519	ATPases with chaperone activity, ATP-binding subunit	NS	-1.08	NS	NS
BL0520	DnaJ-class molecular chaperone	NS	-1.28	NS	NS
BL1250	Molecular chaperone	NS	-1.72	NS	NS

BL1558	10 kDa chaperonin GROES	NS	NS	NS	0.98
BLD_0001	Cold shock protein	NS	NS	1.51	1.06
BL1664	Universal stress protein UspA and related nucleotide-binding proteins	-1.15	-1.74	NS	NS
BLD_1771	Stress-responsive transcriptional regulator PspC	0.84	NS	NS	NS

[a]NS, Not Significant

(Table 6-2). Examples include several genes involved in the thioredoxin reductase system which, under favorable growth conditions, functions with ribonucleoside reductase to use NADPH to reduce the 2' OH group of ribose for deoxynucleotide production, as well as to maintain cytoplasmic redox for disulfide bond production in proteins (25, 26). During oxidative stress, however, cells may use thioredoxin reductase and peroxiredoxin to direct NADPH toward the removal of oxidative free radicals via the reduction of H_2O_2 and toxic lipid hydroperoxides (27, 28, 29). Schell et al. (30) suggested these enzymes might be one of the primary defense mechanisms against oxidative stress in bifidobacteria, and other research has shown up-regulation of thioredoxin, thioredoxin reductase and peroxiredoxin in response to oxygen stress (31, 32). *B. longum* NCC2705 showed significant up regulation of thioredoxin reductase (BL0614) and peroxiredoxin (BL0615) along with ribonucleotide reductase alpha and beta subunits (BL0670, BL0671) after only 5 min sublethal H_2O_2 treatment (Table 6-2). In contrast, D2957 did not display significant upregulation of these genes until 60 min exposure to H_2O_2. These results

confirm that thioredoxin reductase and peroxiredoxin provide a primary defense mechanism against oxidative stress in *B. longum* and suggest that the greater H_2O_2 resistance of strain NCC2705 versus D2957 may be tied to earlier induction of these mechanisms.

Oxidative stress causes protein damage and denaturation, and protection of proteins and processing of damaged proteins is an important component of the oxidative stress response in bacteria (33, 34, 35). Although bifidobacteria possess well studied chaparones and chaparonins (eg. DnaJ/DnaK and GroEL/GroES), their role in H_2O_2 resistance is unclear. *B. longum* NCC2705 for example, showed significant down regulation of several general stress response genes, including DnaJ, UspA and several chaperone proteins (BL0519, BL1250 and BL1664) in response to H_2O_2 exposure (Table 6-2). This observation is consistent with prior studies, which reported no change in expression or down regulation of the general stress response genes in response to acid stress (36, 37). However, NCC2705 did show an up regulation of several proteases (BL0555, BL0781, BL0945) after 5 min H_2O_2 exposure, including an ATP-dependent metallo-protease (BL1682). Jin et al. (36) also reported upregulation of a similar metallo-protease during acid stress, which was hypothesized to function in response to damage of membrane proteins which result in perturbation of membrane function (38). In contrast, strain D2957 only showed upregulation of two proteases (BL0944, BL0945) after 60 min exposure.

Membrane fatty acid analysis. Bacterial cell envelopes provide an initial line of defense against environmental stress and their lipid composition plays a

crucial role in functionality and cell protection (39-43). Membrane fatty acid analysis of *B. longum* strains NCC2705 and D2957 after 5 min (T1) or 60 min (T2) 1.25mM H_2O_2 exposure showed NCC2705 had a significant increase in both $C_{16:0}$ and $C_{18:1n9}$, but a decrease in the $C_{18:1}$ plasmalogen after exsposure compared to cells grown in control media (Fig 6-3). Strain D2957 showed a similar increase in $C_{18:1n9}$ and decrease in $C_{18:1}$ plasmalogen after exposure to 2.55mM H_2O_2 , but no change in $C_{16:0}$ (Fig 6-3).

Direct comparison between the two strains showed they maintain a similar membrane composition, with a very low saturated to unsaturated fatty acid ratio and that the majority of the unsaturated fatty acids occur as $C_{18:1}$ plasmalogens (22). These vinyl ether-linked lipids are more easily oxidized at the carbon oxygen ether bond, and do not propagate free radicals when oxidized compared to their ester linked counterparts (44, 45). Interestingly both strains had low amounts of cyclopropyl fatty acids, which are suggested to decrease the amount of lipid peroxidation in cell membranes by stabilizing the unsaturated bond by addition of a methyl group (46).

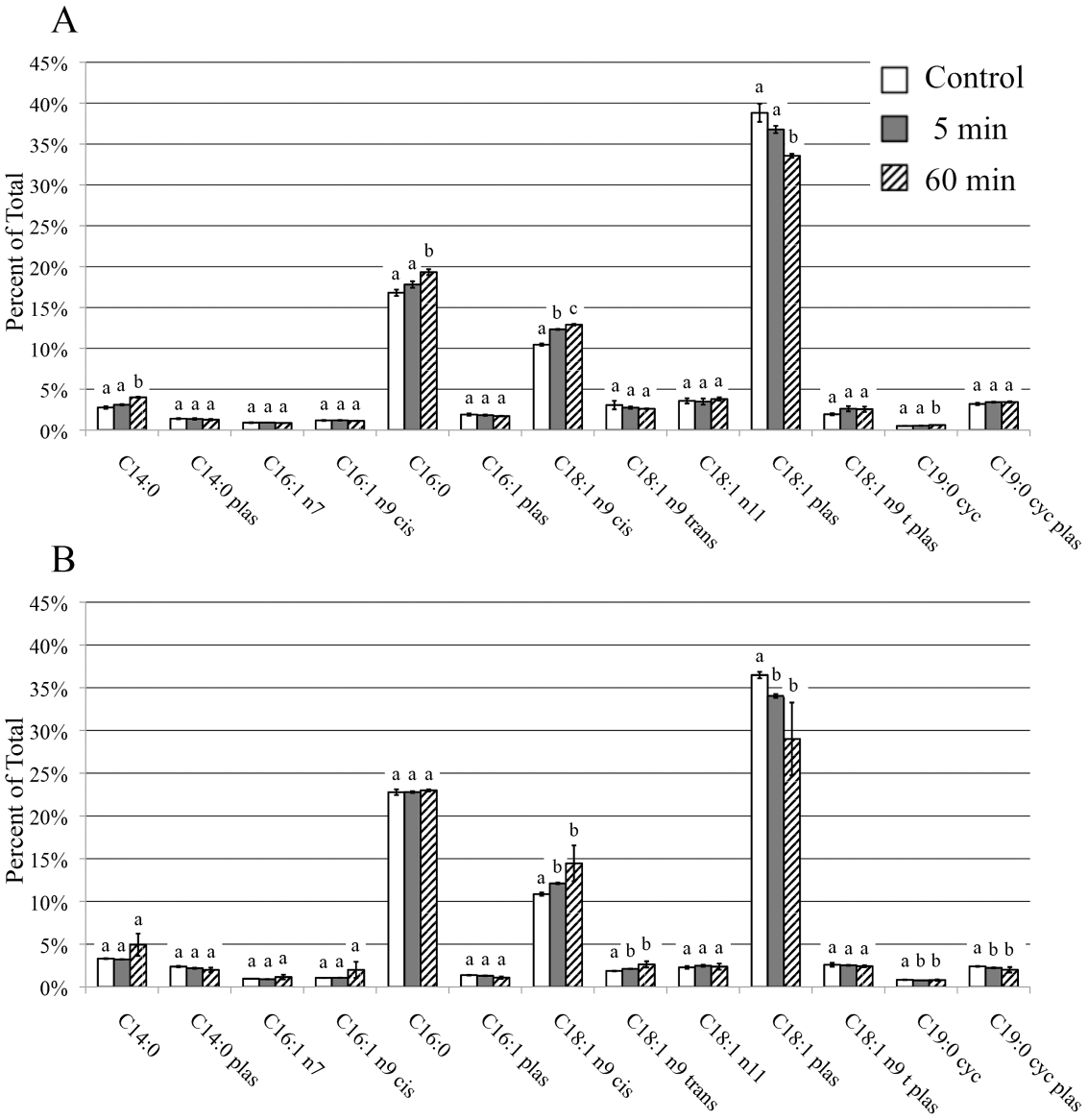

FIG 6-3 Membrane fatty acid composition for *B. longum* NCC2705 (A) and *B. longum* D2957 (B). The graphs shows data from cells grown in MP5 media with no H_2O_2 (control) and cells exposed to 1.25 mM H_2O_2 in MP5 broth for 5 or 20 min. Error bars correspond to the standard error of the mean (SEM). Means with the same letters within each strain are not significantly different ($P < 0.05$).

In summary, transcriptome data suggest that the significant differences in the intrinsic and inducible resistance to H_2O_2 noted previously between *B. longum* NCC2705 and D2957 (16), may be largely due to the timing and degree of induction of genes involved in an oxidative stress response. These data demonstrate that NCC2705 has a rapid and highly inducible H_2O_2 stress response, whereas strain D2957 showed a more delayed and less pronounced transcriptional response to H_2O_2 stress. Because of the highly reactive nature of oxidative free radicals, an immediate and robust inducible stress response may be more effective in neutralizing the damaging effects of H_2O_2.

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CHAPTER 7

SUMMARY AND CONCLUSIONS

This study focused on defining the physiological and transcriptional stress responses of *Bifidobacterium* species to H_2O_2 stress. The fist phase of the study determined the intrinsic and inducible H_2O_2 stress responses of 3 strains of *B. longum* and 3 strains of *B. animalis* subsp. *lactis.* Results from this phase indicate that intrinsic and inducible H_2O_2 resistance is both species and strain specific. Our study design sought to explore H_2O_2 resistance of each strain in a milk peptone-based growth medium similar to those used for commercial production of probiotic cultures to study cells in active metabolism during exposure. Because H_2O_2 is a strong oxidizing agent, we anticipated there would be a loss in concentration upon addition to broth medium and preliminary tests confirmed the concentration of H_2O_2 decreased from 20-60%. Because of this variability, our data analysis for intrinsic resistance was calculated similar to a Z-value determination in thermal destruction of an organism. The measured concentrations of H_2O_2 were plotted against the $\log_{10}$CFUof surviving cells and a line was fitted to the data. A steeper slope of the line indicates faster cell death at higher concentrations of H_2O_2.

Although certain strains showed higher H_2O_2 resistance than others, the lethal H_2O_2 concentration for all strains was relatively low (2.55-5.25 mM), which are within the concentration range of H_2O_2 produced by LAB starter cultures during manufacture of yogurt and other bioactive foods. Our results suggest that a sublethal H_2O_2 exposure could be used to enhance H_2O_2 resistance of some strains

(e.g. *B. longum* NCC2705 and *B. animalis* subsp. *lactis* BL-04), but that H_2O_2 inducible stress responses are not only species and strain dependent, but also dependent on the exposure time and concentration. This can be seen by the fact that *B. animalis* subsp. *lactis* BL-04 showed an increase in survival after a 20 min induction but not after a 60 min induction. It was also interesting to note that sublethal H_2O_2 exposure increased some strains sensitivity to lethal H_2O_2 concentrations in some strains, as was the case for *B. animalis* subsp. *lactis* DSM10140.

Initial analysis of membrane fatty acid compositions for *Bifidobacterium* strains revealed the possibility that a significant percentage of the membrane phospholipids contained ether bound lipids, more commonly referred to as plasmalogens. Previous research on membrane composition during stress exposure in bifidobacteria has not typically taken these ether-bonded lipids into account. Our collected data show that plasmalogens make up a significant proportion of the total membrane composition of *B. animalis* subsp. *lactis* DSM10140 and BL-04 strains. Isolated membrane fatty acids were analysed using GC-MS accurate mass for positive identification of ether-bonded lipids. This data represent the first published gas chromatograms and mass spectrometry tables for plasmalogens isolated from bifidobacteria. Because plasmalogens have been shown to have physical attributes that affect membrane physiology differently compared to the ester linked analogs, it is important to consider these lipids when characterizing the membrane composition of bifidobacteria. The high concentrations of plasmalogens in *B. animalis* subsp. *lactis* membranes, together with strain-specific differences in

lipid species correlated with H_2O_2 sensitivity, suggest these lipids may play an important role in environmental stress resistance.

The second phase of this study explored the transcriptional stress responses of bifidobacteria to H_2O_2 using DNA microarrays. Transcriptome data show a sublethal H_2O_2 exposure triggers induction of an oxidative stress response in *B. animalis* subsp. *lactis* BL-04, but that this mechanism is somehow impaired in strain DSM 10140. Comparison of the whole genome sequences *B. animalis* subsp. *lactis* BL-04 and DSM 10140 reveal that they are highly clonal, yet display significant differences in their intrinsic and inducible resistance to H_2O_2. Genetic and membrane lipid data suggest some of the differences in H_2O_2 resistance between these cells may be associated with activity of a long chain fatty acyl-CoA ligase gene which activates long chain fatty acids for integration into the membrane. While efforts to restore an inducible H_2O_2 stress response in DSM 10140 via modification of its CMFA composition were unsuccessful, modification did significantly increase intrinsic H2O2 resistance. These data show deliberate H_2O_2 stress induction or membrane lipid modification can be used to significantly improve H_2O_2 resistance in *B. animalis* subsp. *lactis*.

Transcriptome data for *B. longum* suggest that the significant differences in the intrinsic and inducible resistance to H_2O_2 between strains NCC2705 and D2957, may be largely due to the timing and degree of induction of genes involved in an oxidative stress response. These data demonstrate that NCC2705 has a rapid and highly inducible H_2O_2 stress response, whereas strain D2957 showed a more

delayed and less pronounced transcriptional response to H_2O_2 stress. Because of the highly reactive nature of oxidative free radicals, an immediate and robust inducible stress response may be more effective in neutralizing the damaging effects of H_2O_2.

Because genetic tools are only poorly developed in *B. animalis* subsp. *lactis* and *B. longum* strains, confirmation of the functional roles of stress response systems identified in this study was not feasible. To truly understand these mechanisms will require functional studies involving genetic manipulation of *B. animalis* subsp. *lactis*, and *B. longum*. Further research could be conducted to develop reliable genetic manipulation techniques in bifidobacteria. These methods could then be used to create functional knockouts to understand the role of the thioredoxin reductase system and the biochemistry behind the synthesis of plasmalogens to help understand their role in membrane physiology and environmental stress adaptation of bifidobacteria.

APPENDICES

APPENDIX A

DATA NOT INCLUDED IN TEXT

TABLE A-1 Differentially regulated genes of *B. lactis* BL-04 during H_2O_2 stress.

General functional category and gene ID	Predicted function	Fold change vs. control	
		T1	T2
Metabolism			
Energy production, conversion			
Balac_1082	Pyruvate formate-lyase activating enzyme	1.46	NS[a]
Balac_0124	Predicted nucleoside-diphosphate-sugar epimerase / NAD-dependent epimerase/dehydratase	1.13	NS
Balac_1561	[phosphocarrier protein HPr]-phosphatase / halo acid dehalogenase-like hydrolase	0.65	NS
Balac_0538	Citrate synthase	0.50	NS
Balac_0768	Isocitrate dehydrogenase [NADP]	-0.46	NS
Balac_0345	Phosphate transport system permease protein pstA	-0.80	NS
Balac_0459	Protein tyrosine phosphatase	-1.28	NS
Balac_0378	Alcohol dehydrogenase / Acetaldehyde dehydrogenase [acetylating]	-2.67	NS
Carbohydrate transport, metabolism			
Balac_1597	Raffinose transport system permease protein	3.46	NS
Balac_1598	Raffinose transport system permease protein	3.20	NS
Balac_1599	Raffinose-binding protein	3.12	NS
Balac_1601	Alpha-galactosidase	2.10	NS
Balac_1567	4-alpha-glucanotransferase	3.13	NS
Balac_1568	Alpha-glucosidase	1.43	NS
Balac_1569	Multiple sugar transport system permease protein msmG	2.58	NS
Balac_1570	Sugar transport system permease protein	2.55	NS
Balac_1572	Maltose/maltodextrin-binding protein	1.90	NS
Balac_1573	Trehalose-6-phosphate hydrolase	0.99	NS
Balac_1593	Oligo-1,6-glucosidase	2.34	NS
Balac_1562	Pullulanase	0.96	NS
Balac_1563	Maltose transport system permease protein malG	0.67	NS
Balac_1564	Maltodextrin transport system permease protein malC	0.59	NS
Balac_0483	MalE-type ABC sugar transport system	-0.68	NS

	periplasmic component		
Balac_0977	Isoamylase / glycogen operon protein GlgX	-0.68	NS
Balac_0065	Alpha-L-arabinofuranosidase	-0.79	NS
Amino acid transport, metabolism			
Balac_0217	2-isopropylmalate synthase	0.88	NS
Balac_0241	3-isopropylmalate dehydratase large subunit	0.86	NS
Balac_0444	Amino acid permease	0.77	NS
Balac_1332	N-acetylglutamate synthase	0.76	NS
Balac_0442	Glutamate/gamma-aminobutyrate antiporter	0.74	-1.37
Balac_1592	Threonine dehydratase	0.68	NS
Balac_1362	3-isopropylmalate dehydrogenase	0.59	NS
Balac_0505	O-acetyl-L-homoserine sulfhydrolase / O-acetyl-L-serine sulfhydrolase	0.49	NS
Balac_0880	Aspartate carbamoyltransferase	-0.48	NS
Balac_0672	Glutamate transport system permease protein gluC/ ABC-type amino acid transport system permease component	-0.65	NS
Balac_0559	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	-0.84	NS
Balac_0560	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	-0.79	NS
Balac_0561	ABC-type dipeptide/oligopeptide/nickel transport system, ATPase component	-0.74	NS
Balac_1201	Phospho-N-acetylmuramoyl-pentapeptide-transferase	-1.01	NS
Balac_1242	ABC-type nitrate/sulfonate/bicarbonate transport system, permease component	-1.59	-1.79
Balac_1243	ABC-type nitrate/sulfonate/bicarbonate transport systems, periplasmic components	-1.81	-2.20
Nucleotide transport, metabolism			
Balac_1501	Sugar kinases, ribokinase family	3.03	2.41
Balac_1503	Inosine-uridine preferring nucleoside hydrolase	2.79	2.54
Balac_1081	Ribonuclease D	1.25	NS
Balac_0946	Xanthine phosphoribosyltransferase	1.14	NS
Balat_0464	5'-nucleotidase	1.05	NS
Balac_0785	Uracil DNA glycosylase superfamily protein	0.83	NS
Balac_0112	O6-methylguanine-DNA methyltransferase	0.82	NS
Balac_0529	Oligoribonuclease	0.57	NS
Balac_0595	Phosphoribosylformylglycinamidine cyclo-	-0.90	NS

	ligase		
Balac_0814	Phosphoribosylaminoimidazolecarboxamide formyltransferase / IMP cyclohydrolase	-0.90	NS
Balac_0874	Orotate phosphoribosyltransferase	-1.05	NS
Balac_0875	Dihydroorotate dehydrogenase	-1.06	NS
Balac_0876	Dihydroorotate dehydrogenase electron transfer subunit	-0.94	NS
Balac_0877	Orotidine 5'-phosphate decarboxylase	-0.96	NS
Balac_0878	Dihydroorotase	-0.83	NS
Balac_0922	CTP synthase	-1.03	NS
Balac_0627	Orotidine 5'-phosphate decarboxylase	-1.20	NS
Balac_0628	Guanylate kinase	-1.26	NS
Balac_1438	Inosine-uridine nucleoside N-ribohydrolase	NS	-1.25
Balac_1244	Inosine-uridine preferring nucleoside hydrolase	-1.78	-2.26
Coenzyme metabolism			
Balac_1405	ABC-type transport system involved in cytochrome b biosynthesis, ATPase and permease components	NS	1.29
Balac_1397	pyridoxine biosynthesis protein	0.76	NS
Information Storage and Processing			
Translation, ribosomal structure, biogenesis			
Balac_0618	SSU ribosomal protein S7P	-0.64	NS
Balac_0350	LSU ribosomal protein L12P (L7/L12)	-0.92	NS
Balac_0760	Ribosomal-protein-S18-alanine acetyltransferase	-0.94	NS
Transcription			
Balac_1571	Transcriptional regulator, LacI family	2.29	NS
Balac_1275	Histone acetyltransferase HPA2 and related acetyltransferases	NS	1.73
Balac_0477	LacI-type transcriptional regulator	0.63	NS
Balac_0064	Hypothetical protein / RbsR-type transcription regulator/ ribose operon repressor	-0.86	NS
DNA Replication, recombination, repair			
Balac_1304	ATP-dependent helicase / CRISPR-associated helicase Cas3 family protein	0.58	NS

Balac_0006	Type IIA topoisomerase (DNA gyrase/topo II, topoisomerase IV), A subunit	NS	-1.03
Cellular Processes			
Protein turnover, stress response			
Balac_0326	Ribonucleoside-diphosphate reductase beta chain	5.39	2.77
Balac_0327	Ribonucleoside-diphosphate reductase alpha chain	4.23	NS
Balac_0328	NrdI protein / ribonucleotide reductase stimulatory protein	1.71	NS
Balac_1502	tetracycline resistance permease/tetracycline efflux pump/MFS[b] transporter/	2.98	2.53
Balac_0865	Peroxiredoxin AhpC	1.10	NS
Balac_0866	Thioredoxin reductase / Thioredoxin/Glutaredoxin family protein	1.84	1.19
Balac_0118	Oxidoreductase	1.40	NS
Balac_0120	Vanillate O-demethylase oxidoreductase / ferric reductase	1.59	NS
Balac_0121	Flavodoxin	2.14	NS
Balac_0123	Flavodoxin	1.54	NS
Balac_0331	Peptidase family U32	1.35	NS
Balac_0443	Carboxypeptidase S1	1.28	-1.27
Balac_0573	NTP pyrophosphohydrolases including oxidative damage repair enzymes	NS	1.34
Balac_1314	Anaerobic ribonucleoside-triphosphate reductase activating protein	1.05	NS
Balat_0226	Dipeptidase A	0.81	1.54
Balac_0440	Acyl-coenzyme A:6-aminopenicillanic-acid-acyltransferase precursor	NS	-1.63
Balac_0441	Aminopeptidase C	0.76	NS
Balac_1315	Anaerobic ribonucleoside-triphosphate reductase	0.72	NS
Balac_1212	LexA repressor	0.60	NS
Balac_1114	RecA protein	0.58	NS
Balac_0025	Oxidoreductase	0.56	NS
Balac_0856	Excinuclease ABC subunit A	0.55	NS
Balac_1437	Multidrug resistance protein B	0.52	-1.22
Balac_1556	GrpE protein	0.47	NS
Balac_1247	DNA repair protein recO	-0.97	NS
Balac_1555	DnaJ-class molecular chaperone	NS	-1.34
Cell secretion			

Balac_0308	Protein translocase subunit secE	0.60	NS
Balac_1110	Protein translocase subunit secA	-1.07	NS
Ion transport			
Balac_0182	High-affinity zinc uptake system protein znuA precursor / Hypothetical exported protein	2.22	NS
Balac_1036	Fe2+ transport system protein B	NS	1.88
Balac_0014	Large-conductance mechanosensitive channel	0.99	NS
Balac_0348	Manganese-binding protein	0.84	NS
Balac_0991	Manganese transport system ATP-binding protein	0.79	NS
Balac_0917	ATP-dependent transporter sufC	0.55	NS
Balac_0919	ABC-type transport system for Fe-S cluster assembly permease component sufB	0.67	NS
Cell envelope biogenesis			
Balac_0261	Fibronectin-binding protein	NS	1.18
Balac_1132	O-acetyltransferase (cell wall biosynthesis)	1.15	NS
Balac_0316	Propionyl-CoA carboxylase beta chain	-0.84	NS
Balac_0317	Fatty acid synthase	-0.77	NS
Balac_1406	Long-chain-fatty-acid--CoA ligase	-0.82	NS
Balac_1380	Flippase Wzx	-0.97	NS
Balac_1387	Capsular polysaccharide synthesis protein	-0.99	NS
Cell division, chromosome partitioning			
Balac_0696	ATPases involved in chromosome partitioning	NS	1.64
Balac_0084	Serine/threonine protein kinase	-0.81	NS
Balac_0085	Serine/threonine protein kinase	-0.81	NS
Balac_0086	Penicillin-binding protein/FtsI	-0.74	NS
Balac_0087	Cell division protein ftsW	-0.68	NS
Balac_0088	Protein phosphatase 2C	-0.82	NS
Balac_1196	Cell division protein ftsQ	-0.74	NS
Poorly characterized, general function only			
Balac_0019	none assigned / membrane-anchored glycerophosphoryl diesterphosphodiesterase-like protein	NS	1.79
Balac_1052	Hypothetical cytosolic protein	1.05	NS
Balac_0868	Predicted hydrolase (HAD superfamily)	NS	1.03
Balac_0325	Hypothetical membrane spanning protein	0.81	NS
Balac_0147	Transporter, MFS superfamily	0.68	NS
Balac_0636	DedA family protein	0.53	NS
Balac_1248	Transporter / Hydrolases of the alpha/beta	-0.84	NS

	superfamily		
Balac_0580	Hypothetical membrane spanning protein	-0.86	-1.66
Balac_0815	Hypothetical membrane spanning protein	-0.90	NS
Balac_1381	Hypothetical protein / glycosyl transferase family protein	-0.96	NS
Balac_0515	Hypothetical protein / putative binding protein-dependent transporter	-1.17	NS
Balac_0516	Transporter / putative binding protein-dependent transporter	-0.98	NS
Balac_0139	Hypothetical protein / membrane protein with transport function	-1.23	NS
Balac_0097	Integral membrane protein	-1.46	-3.11
Uncharacterized, hypothetical proteins			
Balac_0119	Hypothetical protein	1.68	NS
Balac_1037	Hypothetical protein	NS	1.66
Balac_1133	Hypothetical protein	1.15	NS
Balac_1558	Hypothetical protein	1.12	NS
Balac_1275	Hypothetical protein	1.00	NS
Balac_0355	Hypothetical protein	0.68	NS
Balac_0074	Hypothetical cytosolic protein	0.67	NS
Balac_1340	Hypothetical protein	0.67	NS
Balac_0094	Hypothetical protein	0.62	NS
Balac_1323	Hypothetical protein	0.50	NS
Balac_0648	Hypothetical protein	-0.73	NS
Balac_1468	Hypothetical protein	-0.76	NS
Balac_1409	Hypothetical protein	-0.79	NS
Balac_0650	Hypothetical protein	-0.83	NS

[a]NS, Not Significant

[b]MFS, Mutli-facilitator superfamily

Table A-2 Sequenced genes from *B. longum* D2957 predicted to not hybridize on DNA microarray.

General functional category and gene ID	Predicted Function
Metabolism	
Energy production and conversion	
I118_0104	Phosphoenolpyruvate carboxylase
I118_0928	Alcohol dehydrogenase
I118_1667	Ribokinase
I118_1714	Radical SAM, Pyruvate-formate lyase-activating enzyme like
Carbohydrate transport, metabolism	
I118_0011	Alpha-glucosidase
I118_0012	ABC-type sugar transport system, permease component
I118_0013	Multiple sugar ABC transporter, membrane-spanning permease protein MsmF
I118_0015	Multiple sugar ABC transporter, substrate-binding protein
I118_0017	Multiple sugar ABC transporter, substrate-binding protein
I118_0018	MSM (multiple sugar metabolism) operon regulatory protein
I118_0019	MSM (multiple sugar metabolism) operon regulatory protein
I118_0052	Beta-glucosidase
I118_0081	Putative pectinesterase
I118_0291	Maltose/maltodextrin ABC transporter, substrate binding periplasmic protein MalE
I118_0293	ABC-type sugar transport system, permease component
I118_0448	Arabinose permease
I118_0450	Arabinose-proton symporter
I118_0454	Lactose and galactose permease, GPH translocator family

I118_0627	Beta-glucuronidase
I118_1225	Cytidylate kinase / GTP-binding protein EngA
I118_1428	Predicted glucose transporter in maltodextrin utilization gene cluster
I118_1563	Alpha-xylosidase
I118_1663	Lactose and galactose permease, GPH translocator family
I118_1664	Beta-galactosidase
I118_1926	Sucrose phosphorylase
I118_1999	Rhamnose-containing polysacharide translocation permease
I118_2058	Endo-1,4-beta-xylanase

Amino acid transport, metabolism

I118_0702	L-serine dehydratase
I118_1182	N-formylglutamate deformylase
I118_1536	Methionine ABC transporter ATP-binding protein
I118_1896	Methionine gamma-lyase
I118_1945	Aspartate aminotransferase

Nucleotide transport, metabolism

I118_0718	Competence protein F homolog protein YhgH required for utilization of DNA as sole source of carbon and energy
I118_1142	3', 5' oligoribonuclease (orn)
I118_1483	ATP-dependent nuclease, subunit A
I118_1484	ATP-dependent nuclease, subunit B
I118_1599	3', 5'-cyclic-nucleotide 3', 5'-phosphodiesterase / 3', 5'-nucleotidase
I118_1600	3', 5'-cyclic-nucleotide 3', 5'-phosphodiesterase / 3', 5'-nucleotidase
I118_1669	Inosine-uridine preferring nucleoside hydrolase

Coenzyme metabolism

I118_0292	Inositol transport system permease protein
I118_1996	Rhamnosyltransferase

Secondary metabolites, biosynthesis, transport, catabolism

I118_1917	ABC-type bacteriocin/lantibiotic exporters, contain an N-terminal double-glycine peptidase domain

Information Storage and Processing

Translation, ribosomal structure, biogenesis

I118_1398	Translation initiation factor 3
I118_1637	DNA-directed RNA polymerase specialized sigma subunit
I118_1952	Cytoplasmic axial filament protein CafA and Ribonuclease G

Transcription

I118_0156	LacI-type transcriptional regulator
I118_0254	Transcriptional regulator, LysR family
I118_0290	LacI-type transcriptional regulator
I118_0461	Transcriptional regulator
I118_0617	MarR-type transcriptional regulator
I118_0619	Transcription regulator
I118_1558	TetR-type transcriptional regulator
I118_1568	Transcriptional regulators
I118_1671	Transcriptional regulator
I118_1928	LacI-type transcriptional regulator

DNA Replication, recombination, repair

I118_0105	ATPase involved in DNA repair
I118_0146	DNA/RNA helicase of DEAD/DEAH box family
I118_0147	Mutator mutT protein (7,8-dihydro-8-oxoguanine-triphosphatase)

0I118_0639	Replication protein
I118_0999	Adenine-specific methyltransferase
I118_1123	DNA polymerase III beta subunit
I118_1137	putative helicase
I118_1138	putative helicase
I118_1187	Adenine-specific methyltransferase EcoRI
I118_1196	Replicative DNA helicase
I118_1485	DNA polymerase III alpha subunit
I118_1670	GCN5-related N-acetyltransferase
I118_1694	Histone acetyltransferase HPA2 and related acetyltransferases
I118_2047	DNA-damage-inducible protein D
I118_2050	ATP-dependent DNA helicase RecG
I118_0631	Type I restriction-modification system, specificity subunit S
I118_0951	Putative DNA-binding protein in cluster with Type I restriction-modification system
I118_1011	Putative DNA methyltransferase
I118_1012	Type II restriction enzyme, methylase subunit YeeA
I118_1013	Putative DNA methyltransferase
I118_1189	EcoRI methylase/methyltransferase
I118_1656	Type I restriction-modification system, specificity subunit S
I118_1657	Type I restriction-modification system, specificity subunit S
I118_1658	Type I restriction-modification system, DNA-methyltransferase subunit M
I118_1659	Type I restriction-modification system, restriction subunit R

I118_2057 ATP-dependent DNA helicase

Mobile DNA Elements and Plasmids

I118_0628 Phage-related integrase

I118_0629 Mobile element protein

I118_0758 Transposase IS66

I118_0759 Transposase IS66

I118_0915 Mobile element protein

I118_0919 Mobile element protein

I118_0996 Phage-related integrase

I118_0997 Phage-related integrase

I118_1038 Mobile element protein

I118_1341 Transposase and inactivated derivatives

I118_1342 Transposase and inactivated derivatives

I118_1738 Transposase

I118_2002 Mobile element protein

I118_2003 Mobile element protein

I118_2004 Mobile element protein

Cellular Processes

Protein turnover, stress response

I118_0255 Flavodoxin

I118_0754 ATP-dependent Zn protease

I118_0761 Arsenate reductase

I118_0762 Arsenical-resistance protein ACR3

I118_0930 Oxidoreductase

I118_1427 Lactoylglutathione lyase and related lyases

I118_1744 Oxidoreductase of aldo/keto reductase family, subgroup 1

Signal transduction

I118_0921 ADP-ribosylglycohydrolase

I118_1320 GTP pyrophosphokinase , (p)ppGpp synthetase I

Cell secretion

I118_0158 Twin-arginine translocation protein TatC

I118_0159 Twin-arginine translocation protein TatA

I118_1077 Preprotein translocase subunit SecG

I118_1079 Preprotein translocase subunit SecE

Ion transport

I118_0449 Transport ATP-binding protein CydCD

I118_0682 Na+-dependent transporters of the Snf family

Cell envelope biogenesis

I118_0080 Flagellar hook-length control protein FliK

I118_0931 Phospholipase/carboxylesterase

I118_1111 Sortase A, LPXTG specific

I118_1556 Protein similar to YcbI of B. subtilis

I118_1773 N-Acetyl-D-glucosamine permease 2, possible

I118_1994 Glycosyltransferase

I118_1997 Glycosyltransferase

I118_2000 Teichoic acid export ATP-binding protein TagH

I118_2001 putative LicD-family phosphotransferase

I118_2010 1,4-beta-N-acetylmuramidase

I118_2014 Glycosyltransferase

Cell division, chromosome partitioning

I118_1482 Cell division protein FtsZ

Poorly characterized, general function only

I118_0039 Cell wall surface anchor family protein

I118_0090 ATPase

I118_0160 Secreted protein

I118_0294 Putative glycosyl hydrolase of unknown function (DUF1680)

I118_0455 Possible arabinogalactan endo-beta-galactosidase or galactanase

I118_0757 Permeases of the major facilitator superfamily

I118_0971 DUF1526 domain-containing protein

I118_0974 Uncharacterized protein conserved in bacteria

I118_1010 HipA protein

I118_1028 Bifidobacterial FemAB-like protein type 4

I118_1039 Putative virulence protein

I118_1049 Beta-propeller domains of methanol dehydrogenase type

I118_1050 LemA family protein

I118_1071 Putative virulence protein

I118_1172 NLP/P60 family protein

I118_1296 Membrane proteins related to metalloendopeptidases

I118_1431 Macro domain, possibly ADP-ribose binding module

I118_1486 Predicted transcriptional regulator

I118_1564 Extracellular solute-binding protein, family 1

I118_1681 O-Glycosyl hydrolase family 30

I118_1995 Integral membrane protein

I118_1998 Probable glucosyltransferase

Uncharacterized, hypothetical proteins

I118_0010 hypothetical protein
I118_0014 hypothetical protein
I118_0016 hypothetical protein
I118_0027 hypothetical protein
I118_0037 hypothetical protein
I118_0050 hypothetical protein
I118_0061 hypothetical protein
I118_0089 hypothetical protein
I118_0100 hypothetical protein
I118_0101 hypothetical protein
I118_0106 hypothetical protein
I118_0107 hypothetical protein
I118_0108 hypothetical protein
I118_0112 hypothetical protein
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I118_0161 hypothetical protein
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I118_0179 hypothetical protein
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I118_0196 hypothetical protein
I118_0217 hypothetical protein
I118_0223 hypothetical protein
I118_0248 hypothetical protein
I118_0249 hypothetical protein
I118_0250 hypothetical protein
I118_0258 hypothetical protein
I118_0295 hypothetical protein
I118_0296 hypothetical protein
I118_0297 hypothetical protein
I118_0317 hypothetical protein
I118_0323 hypothetical protein
I118_0334 hypothetical protein
I118_0336 hypothetical protein
I118_0355 hypothetical protein

I118_0370	hypothetical protein
I118_0387	hypothetical protein
I118_0422	hypothetical protein
I118_0462	hypothetical protein
I118_0463	hypothetical protein
I118_0464	hypothetical protein
I118_0475	hypothetical protein
I118_0479	hypothetical protein
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I118_0717	hypothetical protein
I118_0741	hypothetical protein
I118_0755	hypothetical protein
I118_0756	hypothetical protein
I118_0760	hypothetical protein
I118_0764	hypothetical protein
I118_0804	hypothetical protein
I118_0809	hypothetical protein
I118_0811	hypothetical protein
I118_0826	hypothetical protein

I118_0827	hypothetical protein
I118_0832	hypothetical protein
I118_0836	hypothetical protein
I118_0860	hypothetical protein
I118_0892	hypothetical protein
I118_0895	hypothetical protein
I118_0902	hypothetical protein
I118_0914	hypothetical protein
I118_0920	hypothetical protein
I118_0922	hypothetical protein
I118_0923	hypothetical protein
I118_0924	hypothetical protein
I118_0925	hypothetical protein
I118_0926	hypothetical protein
I118_0927	hypothetical protein
I118_0945	hypothetical protein
I118_0952	hypothetical protein
I118_0953	hypothetical protein
I118_0954	hypothetical protein
I118_0956	hypothetical protein
I118_0966	hypothetical protein
I118_0967	hypothetical protein
I118_0969	hypothetical protein
I118_0972	hypothetical protein
I118_0982	hypothetical protein
I118_0998	hypothetical protein
I118_1000	hypothetical protein
I118_1001	hypothetical protein
I118_1002	hypothetical protein
I118_1003	hypothetical protein
I118_1004	hypothetical protein
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I118_1006	hypothetical protein
I118_1007	hypothetical protein
I118_1008	hypothetical protein
I118_1009	hypothetical protein
I118_1014	hypothetical protein
I118_1015	hypothetical protein
I118_1016	hypothetical protein
I118_1017	hypothetical protein
I118_1018	hypothetical protein
I118_1019	hypothetical protein
I118_1020	hypothetical protein
I118_1021	hypothetical protein

I118_1022	hypothetical protein
I118_1023	hypothetical protein
I118_1024	hypothetical protein
I118_1025	hypothetical protein
I118_1026	hypothetical protein
I118_1027	hypothetical protein
I118_1029	hypothetical protein
I118_1030	hypothetical protein
I118_1031	hypothetical protein
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I118_1063	hypothetical protein
I118_1064	hypothetical protein
I118_1065	hypothetical protein
I118_1066	hypothetical protein
I118_1067	hypothetical protein
I118_1068	hypothetical protein
I118_1069	hypothetical protein
I118_1070	hypothetical protein

I118_1072	hypothetical protein
I118_1073	hypothetical protein
I118_1074	hypothetical protein
I118_1075	hypothetical protein
I118_1076	hypothetical protein
I118_1078	hypothetical protein
I118_1095	hypothetical protein
I118_1102	hypothetical protein
I118_1104	hypothetical protein
I118_1105	hypothetical protein
I118_1106	hypothetical protein
I118_1107	hypothetical protein
I118_1108	hypothetical protein
I118_1109	hypothetical protein
I118_1110	hypothetical protein
I118_1112	hypothetical protein
I118_1113	hypothetical protein
I118_1114	hypothetical protein
I118_1115	hypothetical protein
I118_1116	hypothetical protein
I118_1117	hypothetical protein
I118_1118	hypothetical protein
I118_1119	hypothetical protein
I118_1120	hypothetical protein
I118_1121	hypothetical protein
I118_1122	hypothetical protein
I118_1124	hypothetical protein
I118_1126	hypothetical protein
I118_1127	hypothetical protein
I118_1128	hypothetical protein
I118_1129	hypothetical protein
I118_1130	hypothetical protein
I118_1131	hypothetical protein
I118_1132	hypothetical protein
I118_1133	hypothetical protein
I118_1134	hypothetical protein
I118_1135	hypothetical protein
I118_1136	hypothetical protein
I118_1139	hypothetical protein
I118_1140	hypothetical protein
I118_1141	hypothetical protein
I118_1143	hypothetical protein
I118_1144	hypothetical protein
I118_1145	hypothetical protein

I118_1146	hypothetical protein
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I118_1148	hypothetical protein
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I118_1167	hypothetical protein
I118_1168	hypothetical protein
I118_1169	hypothetical protein
I118_1170	hypothetical protein
I118_1171	hypothetical protein
I118_1173	hypothetical protein
I118_1174	hypothetical protein
I118_1175	hypothetical protein
I118_1176	hypothetical protein
I118_1177	hypothetical protein
I118_1178	hypothetical protein
I118_1179	hypothetical protein
I118_1180	hypothetical protein
I118_1181	hypothetical protein
I118_1183	hypothetical protein
I118_1184	hypothetical protein
I118_1185	hypothetical protein
I118_1186	hypothetical protein
I118_1188	hypothetical protein
I118_1190	hypothetical protein
I118_1191	hypothetical protein
I118_1192	hypothetical protein
I118_1193	hypothetical protein

I118_1194	hypothetical protein
I118_1195	hypothetical protein
I118_1197	hypothetical protein
I118_1198	hypothetical protein
I118_1199	hypothetical protein
I118_1200	hypothetical protein
I118_1201	hypothetical protein
I118_1202	hypothetical protein
I118_1203	hypothetical protein
I118_1204	hypothetical protein
I118_1205	hypothetical protein
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I118_1209	hypothetical protein
I118_1210	hypothetical protein
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I118_1212	hypothetical protein
I118_1213	hypothetical protein
I118_1214	hypothetical protein
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I118_1217	hypothetical protein
I118_1266	hypothetical protein
I118_1318	hypothetical protein
I118_1338	hypothetical protein
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I118_1399	hypothetical protein
I118_1402	hypothetical protein
I118_1430	hypothetical protein
I118_1432	hypothetical protein
I118_1453	hypothetical protein
I118_1476	hypothetical protein
I118_1478	hypothetical protein
I118_1479	hypothetical protein
I118_1480	hypothetical protein
I118_1481	hypothetical protein
I118_1497	hypothetical protein
I118_1504	hypothetical protein
I118_1505	hypothetical protein
I118_1512	hypothetical protein
I118_1537	hypothetical protein

I118_1557	hypothetical protein
I118_1561	hypothetical protein
I118_1567	hypothetical protein
I118_1574	hypothetical protein
I118_1606	hypothetical protein
I118_1614	hypothetical protein
I118_1661	hypothetical protein
I118_1666	hypothetical protein
I118_1668	hypothetical protein
I118_1679	hypothetical protein
I118_1683	hypothetical protein
I118_1684	hypothetical protein
I118_1708	hypothetical protein
I118_1713	hypothetical protein
I118_1715	hypothetical protein
I118_1718	hypothetical protein
I118_1728	hypothetical protein
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I118_1813	hypothetical protein
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I118_1991	hypothetical protein
I118_1993	hypothetical protein
I118_2007	hypothetical protein
I118_2009	hypothetical protein
I118_2011	hypothetical protein
I118_2012	hypothetical protein
I118_2013	hypothetical protein
I118_2016	hypothetical protein
I118_2028	hypothetical protein
I118_2033	hypothetical protein
I118_2038	hypothetical protein
I118_2056	hypothetical protein
I118_2059	hypothetical protein
I118_2060	hypothetical protein

I118_2061	hypothetical protein
I118_2062	hypothetical protein
I118_2070	hypothetical protein

Table A-3 Differentially regulated genes associated with the oxidative stress response of *B. longum* strains.

General functional category and gene ID	Predicted Function	Fold Change vs Control			
		NCC2705		D2957	
		T1	T2	T1	T2
Metabolism					
Energy production, conversion					
BL0279	Glucose-6-phosphate isomerase	0.85	NS[a]	NS	1.07
BL0358	ATP synthase gamma chain	NS	NS	NS	0.87
BL0361	F0F1-type ATP synthase, subunit b	1.06	NS	NS	NS
BL0362	F0F1-type ATP synthase, subunit c	1.03	NS	NS	NS
BL0363	F0F1-type ATP synthase, subunit a	0.95	NS	0.95	1.09
BL0604	Phosphoenolpyruvate carboxylase	1.06	NS	NS	0.97
BL0620	Gluconate kinase	-0.78	NS	NS	NS
BL0707	3-phosphoglycerate kinase	1.55	1.48	NS	1.13
BL0715	Transaldolase	-0.95	NS	NS	NS
BL0732	Succinyl-CoA synthetase, beta subunit	0.72	NS	NS	NS
BL0753	Pentose-5-phosphate-3-epimerase	0.91	NS	NS	NS
BL0884	Fructose-2,6-bisphosphatase	0.85	NS	NS	NS
BL0963	Ribose-phosphate pyrophosphokinase	NS	NS	NS	1.05
BL0969	Acetate kinase	1.04	NS	NS	NS
BL0988	Pyruvate kinase	1.55	1.30	NS	1.35
BL1022	Enolase	1.29	NS	NS	NS
BL1259	Polyphosphate kinase	0.78	NS	NS	NS
BL1308	Malate/lactate dehydrogenases	1.56	1.82	NS	2.23
BL1360	Galactose mutarotase and related enzymes	0.72	NS	NS	NS
BL1575	Alcohol dehydrogenase / Acetaldehyde dehydrogenase (acetylating)	NS	NS	NS	1.35
BL1656	Phosphoglycerate mutase 1	0.74	1.01	NS	1.32
BL1673	Lactaldehyde reductase	NS	-2.30	NS	NS
BL1726	Pyruvate-formate lyase-activating enzyme	-1.57	-1.58	NS	NS
Carbohydrate transport, metabolism					
BL0143	ABC-type sugar transport systems, permease components	1.51	NS	NS	NS

BL0208	ABC-type polysaccharide/polyol phosphate export systems, permease component	0.84	NS	NS	NS
BL0273	Ribulose-5-phosphate 4-epimerase and related epimerases and aldolases	NS	-1.05	NS	NS
BL0420	Beta-xylosidase	-0.73	NS	NS	NS
BL0423	ABC-type sugar transport system, permease component	-0.71	NS	NS	NS
BL0425	ABC-type sugar transport system, periplasmic component	NS	-1.73	NS	-1.66
BL0476	Monosaccharide translocase (flippase type)	0.92	NS	NS	NS
BL0527	4-alpha-glucanotransferase (Maltose Degredation)	1.25	NS	NS	NS
BL0536	Glycosidases	NS	-0.95	NS	NS
BL0597	Glucan phosphorylase	1.86	1.10	1.12	1.25
BL0774	ADP-ribose pyrophosphatase	1.15	0.78	NS	NS
BL0999	1,4-alpha-glucan branching enzyme	1.54	0.92	NS	NS
BL1164	ABC-type sugar transport system, periplasmic component	NS	-2.14	NS	-1.75
BL1168	Beta-galactosidase	-1.24	-2.23	NS	-1.57
BL1169	ABC-type sugar transport system, permease component	-1.49	-2.89	NS	-1.63
BL1170	ABC-type sugar transport systems, permease components	-1.65	-2.82	NS	-1.80
BL1187	Phosphomannomutase	0.87	NS	NS	NS
BL1292	2,5-diketo-D-gluconic acid reductase	NS	NS	NS	0.99
BL1339	Sugar kinases, ribokinase	NS	-1.55	NS	NS
BL1431	Sugar kinases, ribokinase	NS	1.01	NS	NS
BL1443	Cellobiohydrolase A (1,4-beta-cellobiosidase A)	1.00	NS	NS	NS
BL1639	ABC-type sugar transport systems, permease components	1.23	NS	NS	NS
BL1694	ABC-type xylose transport system, periplasmic component	-1.45	-2.68	NS	-2.50
BL1695	ABC-type sugar transport system, ATPase component	-1.64	-2.54	NS	-1.71
BL1696	ABC-type xylose transport system, permease component	-1.18	-2.14	NS	NS

BL1796	Archaeal fructose-1,6-bisphosphatase and related enzymes of inositol monophosphatase family	1.12	NS	NS	NS
BLD_1756	Permeases of the major facilitator superfamily (Arabinose efflux permease)	NS	0.85	NS	NS
BLD_1390	Glucan phosphorylase	1.13	NS	NS	NS
BLD_1323	Trehalose-6-phosphate hydrolase	NS	NS	NS	0.98
BLD_1497	Oligo-1,6-glucosidase	NS	NS	NS	1.51
BLD_1557	ABC-type sugar transport system, ATPase component	1.69	NS	NS	NS
Amino acid transport, metabolism					
BL0074	ABC-type amino acid transport system, permease component	1.03	1.18	NS	1.33
BL0488	2-isopropylmalate synthase	NS	0.95	NS	NS
BL0555	Trypsin-like serine proteases, typically periplasmic	NS	1.37	NS	NS
BL0552	NADPH-dependent glutamate synthase beta chain and related oxidoreductases	0.88	NS	NS	NS
BL0628	Aspartate/tyrosine/aromatic aminotransferase	1.00	1.36	NS	NS
BL0704	Shikimate 5-dehydrogenase	1.06	NS	NS	NS
BL0829	Oligopeptide-binding protein oppA	NS	NS	NS	0.92
BL0869	Cysteine desulfurase / Selenocysteine lyase	NS	NS	NS	0.78
BL1016	FKBP-type peptidyl-prolyl cis-trans isomerases 1	NS	1.35	NS	NS
BL1076	Glutamine synthetase	1.67	NS	1.15	0.89
BL1118	ABC-type amino acid transport/signal transduction systems, periplasmic component/domain	0.90	NS	NS	0.73
BL1119	ABC-type amino acid transport system, permease component	0.86	NS	NS	NS
BL1142	Asparaginase	1.04	0.93	NS	NS
BL1193	Dihydrodipicolinate synthase	NS	NS	NS	0.96
BL1274	Homoserine dehydrogenase	NS	NS	NS	1.16
BL1302	Glutamine synthetase	0.85	NS	NS	NS
BL1350	Xaa-Pro aminopeptidase	0.83	NS	NS	NS
BL1386	ABC-type oligopeptide transport	1.36	NS	NS	1.53

	system, periplasmic component				
BL1387	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	1.25	NS	NS	NS
BL1389	ABC-type dipeptide/oligopeptide/nickel transport systems, permease components	1.14	NS	NS	NS
BL1442	Peptidyl-prolyl cis-trans isomerase (rotamase)	1.30	NS	NS	1.03
BLD_0784	Gamma-glutamyl phosphate reductase	0.94	NS	NS	NS
Nucleotide transport, metabolism					
BL0229	dTDP-D-glucose 4,6-dehydratase	-0.77	NS	NS	NS
BL0444	6-phosphogluconate dehydrogenase	0.80	1.06	NS	NS
BL0731	Adenine/guanine phosphoribosyltransferases	1.00	NS	NS	NS
BL0739	UDP-glucose pyrophosphorylase	1.42	0.97	NS	NS
BL0788	Orotate phosphoribosyltransferase	0.78	1.49	NS	1.18
BL0874	CTP synthase (UTP-ammonia lyase)	1.20	NS	NS	NS
BL0963	Phosphoribosylpyrophosphate synthetase	0.77	NS	NS	NS
BL1107	Phosphoribosylaminoimidazolesuccinocarboxamide (SAICAR) synthase	0.87	NS	NS	1.08
BL1245	UDP-galactopyranose mutase	0.97	NS	NS	NS
BL1392	dUTPase	1.19	NS	NS	NS
BL1437	dUTPase	1.31	1.07	NS	NS
BL1439	Guanosine polyphosphate pyrophosphohydrolases/synthetases	1.09	NS	NS	NS
BL1500	IMP dehydrogenase/GMP reductase	0.90	NS	NS	0.84
BL1505	Uridylate kinase	1.02	1.05	1.00	0.80
BL1681	Hypoxanthine-guanine phosphoribosyltransferase	1.30	1.17	1.09	NS
BL1722	Inosine-5'-monophosphate dehydrogenase	NS	NS	NS	1.39
BL1770	Inosine-uridine nucleoside N-	NS	-2.69	NS	-1.88

	ribohydrolase				
BL1800	Adenylosuccinate lyase	0.96	NS	NS	1.02
BLD_0406	Uridylate kinase	1.06	NS	0.92	0.70
BLD_0702	GMP synthase, PP-ATPase domain/subunit	0.87	NS	NS	0.79
BLD_1589	Uracil phosphoribosyltransferase	1.41	NS	NS	NS
BLD_0976	Dihydroorotate dehydrogenase	NS	NS	NS	0.69
Coenzyme metabolism					
BL0287	Nicotinic acid phosphoribosyltransferase	0.89	1.20	NS	NS
BL0735	AICAR transformylase / IMP cyclohydrolase PurH	1.29	NS	NS	NS
BL1361	4-hydroxy-3-methylbut-2-enyl diphosphate reductase	0.80	NS	NS	NS
BLD_0218	Folylpolyglutamate synthase / Dihydrofolate synthase	NS	NS	NS	0.79
BL1145	Predicted glutamine amidotransferase involved in pyridoxine biosynthesis	0.93	NS	NS	NS
BL1146	Pyridoxine biosynthesis enzyme	1.34	NS	NS	NS
BL1292	Aldo/keto reductases, related to diketogulonate reductase	0.81	NS	NS	NS
Secondary metabolites, biosynthesis, transport, catabolism					
BL0103	ABC-type nitrate/sulfonate/bicarbonate transport systems, periplasmic components	0.77	NS	NS	1.15
BL0155	ABC-type antimicrobial peptide transport system, permease component	-0.86	NS	NS	NS
BL0198	ABC-type antimicrobial peptide transport system, permease component	1.22	NS	NS	NS
BL0251	Permeases of the major facilitator superfamily	-0.78	NS	NS	NS
BL0342	ABC-type spermidine/putrescine transport system, permease component II	-0.81	NS	NS	NS
BL0844	Na+-driven multidrug efflux pump	-0.83	NS	NS	NS
BL0931	ABC-type antimicrobial peptide transport system, permease component	NS	2.11	NS	NS

BL0932	ABC-type antimicrobial peptide transport system, ATPase component	2.00	2.45	NS	NS
BL1041	ABC-type multidrug transport system, ATPase component	-0.92	NS	NS	NS
BL1192	Predicted hydrolase of the metallo-beta-lactamase superfamily	1.15	NS	NS	NS
BL1277	ABC-type antimicrobial peptide transport system, ATPase component	1.38	1.25	NS	NS
BL1631	Permeases of the major facilitator superfamily	1.76	NS	NS	NS
BL1699	Permeases of the major facilitator superfamily	-0.95	NS	NS	NS
BL1771	C4-dicarboxylate transporter	NS	-2.23	NS	NS
BLD_0673	ABC-type antimicrobial peptide transport system, ATPase component	1.65	NS	NS	NS
BLD_1756	Permeases of the major facilitator superfamily	1.66	NS	NS	NS
BLD_1557	ABC-type antimicrobial peptide transport system, ATPase component	NS	0.89	NS	NS
Information Storage and Processing					
Translation, ribosomal structure, biogenesis					
BL0294	LSU ribosomal protein L32P	0.96	1.04	NS	NS
BL0305	Ribosomal protein S16	0.85	NS	NS	NS
BL0330	Ribosomal protein L28	0.99	NS	NS	NS
BL0414	Ribosomal protein S18	0.70	NS	NS	NS
BL0416	Ribosomal protein S6	1.31	0.88	NS	NS
BL0849	Ribosomal protein S20	1.06	NS	NS	NS
BL0853	Ribosomal protein L25	1.27	0.91	NS	NS
BL0886	Ribosomal protein S4 and related proteins	0.82	NS	NS	NS
BL1282	Ribosomal protein L21	1.53	1.16	1.32	0.86
BL1283	Ribosomal protein L27	1.00	NS	NS	NS
BL1366a	LSU ribosomal protein L35P	1.42	NS	NS	NS
BL1367	Ribosomal protein L20	1.02	NS	NS	NS
BL1418	Ribosome-associated protein Y (PSrp-1)	-0.82	-1.34	NS	NS
BL1503	Ribosomal protein S2	1.16	NS	NS	NS
BL1506	Ribosome recycling factor	0.94	NS	NS	0.92
BL1550	LSU ribosomal protein L12P	NS	NS	NS	-1.00

	(L7/L12)				
BL1560	Ribosomal protein L33	1.05	NS	NS	NS
BL1577	Ribosomal protein S10	0.77	NS	NS	NS
BL1586	Ribosomal protein L16/L10E	-0.79	-1.02	NS	NS
BL1588	Ribosomal protein L29	-1.04	-1.31	NS	NS
BL1589	Ribosomal protein S17	-1.00	-1.36	NS	NS
BL1603	Ribosomal protein L36	0.64	NS	NS	NS
BL1605	SSU ribosomal protein S11P	0.89	NS	NS	NS
BL1604	Ribosomal protein S13	0.89	NS	NS	NS
BL0016	tRNA delta(2)-isopentenylpyrophosphate transferase	1.11	1.07	NS	NS
BL0065	Translation elongation factor P (EF-P)/translation initiation factor 5A (eIF-5A)	1.57	1.38	1.17	1.17
BL0286	RNase PH (tRNA processing)	0.80	NS	NS	NS
BL0404	Asp-tRNAAsn/Glu-tRNAGln amidotransferase C subunit	0.72	NS	NS	NS
BL0724	Threonyl-tRNA synthetase	0.82	NS	NS	NS
BL0882	Alanyl-tRNA synthetase	1.04	NS	NS	NS
BL1098	Protein Translation Elongation Factor G (EF-G)	NS	NS	NS	0.98
BL1067	Phenylalanyl-tRNA synthetase alpha chain	NS	NS	NS	0.91
BL1186	N-formylmethionyl-tRNA deformylase	1.08	NS	NS	NS
BL1366	Translation initiation factor 3 (IF-3)	1.36	NS	NS	NS
BL1602	Translation initiation factor 1 (IF-1)	0.94	NS	NS	NS
BL1635	Seryl-tRNA synthetase	0.76	0.90	NS	NS
Transcription					
BL0010	Negative regulator of genetic competence clpC/ Hemolysin TlyB	NS	NS	NS	0.89
BL0047	Transcriptional regulator/sugar kinase	0.79	NS	NS	NS
BL0066	Transcription termination factor	0.89	NS	NS	NS
BL0516	Predicted transcriptional regulators	NS	-1.04	NS	NS
BL0543	Transcriptional regulators	-0.68	NS	NS	NS
BL0746	Predicted transcriptional regulators	1.18	1.05	NS	NS
BL0986	Transcriptional regulator	NS	NS	1.04	NS

BL1011	DNA-directed RNA polymerase specialized sigma subunit, sigma24 homolog	2.16	2.36	NS	NS
BL1015	Transcription elongation factor	1.38	1.27	NS	NS
BL1041a	Predicted transcriptional regulators	-0.88	NS	NS	NS
BL1171	Transcriptional regulator, LacI family	-1.63	-2.38	NS	-1.83
BL1204	DNA-directed RNA polymerase beta' chain	NS	NS	NS	0.69
BL1205	DNA-directed RNA polymerase beta chain	NS	NS	NS	0.80
BL1312	Predicted transcriptional regulator, consists of a Zn-ribbon and ATP-cone domains	0.99	1.03	NS	NS
BL1325	Predicted transcriptional regulator	-1.47	-1.71	NS	NS
BL1357	DNA-directed RNA polymerase specialized sigma subunit, sigma24 homolog	1.67	1.30	NS	NS
BL1358	ECF-type sigma factor negative effector	-0.95	-1.00	NS	NS
BL1414	Predicted transcriptional regulators	0.86	NS	NS	NS
BL1428	DNA-directed RNA polymerase, sigma subunit (sigma70/sigma32)	0.84	NS	NS	NS
BL1504	Translation elongation factor Ts	0.86	NS	NS	NS
BL1574	Transcriptional regulator/sugar kinase	-0.83	NS	NS	NS
BL1769	Histone acetyltransferase HPA2 and related acetyltransferases	NS	-2.58	NS	-2.04
BL1787	DNA-directed RNA polymerase, subunit K/omega	0.98	0.91	NS	NS
BLD_0762	Transcriptional regulator LytR family	1.19	NS	NS	NS
BLD_0925	Transcriptional regulators LacI family	1.15	NS	NS	NS
BLD_1459	Putative transcriptional regulator	-0.83	NS	NS	NS
DNA Replication, recombination, repair					
BL0415	Single-stranded DNA-binding protein	0.70	NS	NS	NS
BL1415	RecA/RadA recombinase	NS	0.88	NS	NS
Mobile DNA Elements and Plasmids					
BL0240	DNA integration/recombination	-0.93	NS	NS	NS

	protein				
BL0241	Integrase/Phage associated	-0.96	NS	NS	NS
BL0246	Integrase/Phage associated	-0.93	NS	NS	NS
BL1779	Integrase/Phage associated	-0.81	NS	NS	NS
BLD_0309	Transposase	-0.77	NS	NS	NS
BLD_0313	Transposase	-0.74	NS	NS	NS
BLD_0995	Transposase and inactivated derivatives	-0.93	NS	NS	NS
BLD_1412	Transposase	-0.85	NS	NS	NS
BLD_0996	DNA replication protein (transposase)	-0.82	NS	NS	NS
BLD_1655	DNA replication protein (transposase)	-0.77	NS	NS	NS
pBLO1_01	Replication protein (on plasmid)	-0.80	NS	NS	NS
BLD_1562	Transposase and inactivated derivatives	-0.79	NS	NS	NS
BLD_1562	Transposase and inactivated derivatives	-0.81	NS	NS	NS
Cellular Processes					
Protein turnover, stress response					
BL0555	Trypsin-like serine proteases, typically periplasmic	1.68	NS	NS	NS
BL0781	Putative intracellular protease/amidase	0.94	0.96	NS	NS
BL0944	ATP-dependent endopeptidase clp proteolytic subunit clpP	NS	NS	NS	0.96
BL0945	Protease subunit of ATP-dependent Clp proteases	0.79	NS	NS	1.48
BL1682	ATP-dependent Zn proteases	0.99	NS	NS	NS
BL0139	NAD(P)H-dependent FMN reductase	NS	NS	NS	1.92
BL0399	Protoporphyrinogen oxidase	1.06	NS	NS	NS
BL0552	NADPH-dependent glutamate synthase beta chain and related oxidoreductases	NS	1.03	NS	NS
BL0614	Thioredoxin reductase	1.92	1.56	NS	0.88
BL0615	Peroxiredoxin	1.36	1.29	NS	1.16
BL0668	Glutaredoxin and related proteins	1.79	1.59	1.57	1.49
BL0669	NrdI protein/ ribonucleotide reductase stimulatory protein	1.42	NS	NS	NS
BL0670	Ribonucleotide reductase, alpha subunit	0.92	NS	NS	NS
BL0671	Ribonucleoside-diphosphate	1.40	NS	NS	0.73

	reductase beta chain				
BL1563	Ferredoxin	0.86	NS	NS	NS
BL1750	Exodeoxyribonuclease VII small subunit	0.75	1.10	NS	NS
BL1752	Oxygen-sensitive ribonucleoside-triphosphate reductase	1.88	1.59	NS	NS
BL1753	Anaerobic ribonucleoside-triphosphate reductase activating protein	0.95	0.89	NS	NS
BLD_0988	Thioredoxin	NS	NS	NS	1.52
BL0001	Cold shock proteins	0.75	NS	NS	0.85
BL0002	60 kDa chaperonin GROEL	NS	NS	NS	1.09
BL0010	ATPases with chaperone activity, ATP-binding subunit	0.89	NS	NS	NS
BL0355	Predicted nuclease of the RecB family	-0.82	NS	NS	NS
BL0517	Molecular chaperone (small heat shock protein)	NS	0.92	NS	NS
BL0519	ATPases with chaperone activity, ATP-binding subunit	NS	-1.08	NS	NS
BL0520	DnaJ-class molecular chaperone	NS	-1.28	NS	NS
BL0576	Universal stress protein UspA (Hsp20 Small Heat Shock Chaparone)	0.98	-1.58	NS	NS
BL1250	Molecular chaperone	NS	-1.72	NS	NS
BL1558	10 kDa chaperonin GROES	NS	NS	NS	0.98
BLD_0001	Cold shock protein	NS	NS	1.51	1.06
BL1664	Universal stress protein UspA and related nucleotide-binding proteins	-1.15	-1.74	NS	NS
BLD_1771	Stress-responsive transcriptional regulator PspC	0.84	NS	NS	NS
Signal transduction					
BL1152	LuxS protein involved in autoinducer AI2 synthesis (quorum sensing)	0.76	1.52	NS	NS
Cell secretion					
BL0709	Preprotein translocase subunit SecG	1.21	1.26	1.25	1.05
BL0730	Protein translocase subunit YajC	1.49	1.61	NS	1.54
BL0982	Type II secretory pathway, pullulanase PulA and related glycosidases	1.24	NS	NS	NS

BL1287	Protein translocase subunit secE	0.84	NS	NS	NS
BL1600	Preprotein translocase subunit SecY	0.70	NS	NS	NS
Ion transport					
BL0872	ABC-type transport system involved in Fe-S cluster assembly, permease component	1.28	1.21	NS	NS
BL1309	Co/Zn/Cd efflux system component	0.77	1.30	NS	0.96
BL1393	K+ transport systems, NAD-binding component	0.79	NS	NS	NS
BLD_0780	Na+-driven multidrug efflux pump	NS	NS	NS	1.02
BLD_0789	Cation transport ATPase	-0.82	NS	NS	NS
BLD_1457	Kef-type K+ transport systems, predicted NAD-binding component	-0.73	NS	NS	NS
Cell envelope biogenesis					
BL0157	Lipopolysaccharide biosynthesis proteins, LPS:glycosyltransferases	-0.89	-1.03	NS	NS
BL0488	Isopropylmalate/homocitrate / citramalate synthases	0.75	NS	NS	NS
BL0672	Glycosyltransferases involved in cell wall biogenesis	-0.75	NS	NS	NS
BL0796	Choloylglycine hydrolase (Bile Salt Hydrolase)	NS	NS	NS	1.62
BL0826	Glycosyltransferase	0.80	NS	NS	NS
BL0858	Long-chain acyl-CoA synthetases (AMP-forming)	1.09	NS	NS	NS
BL1215	Membrane carboxypeptidase (penicillin-binding protein)	0.84	1.19	NS	NS
BL1356	UDP-N-acetylmuramyl tripeptide synthase	0.84	NS	NS	NS
BL1424	1-acyl-sn-glycerol-3-phosphate acyltransferase	1.15	1.20	NS	NS
BL1501	Long-chain acyl-CoA synthetases (AMP-forming)	0.78	NS	NS	NS
BL1575	Alcohol dehydrogenase / Acetaldehyde dehydrogenase [acetylating]	NS	1.19	NS	NS
Cell division, chromosome partitioning					
BL0118	Cell division GTPase	0.73	NS	NS	NS
BL0121	Cell division initiation protein	1.24	NS	NS	NS
BL0322	Cell division initiation protein	1.79	1.52	NS	NS

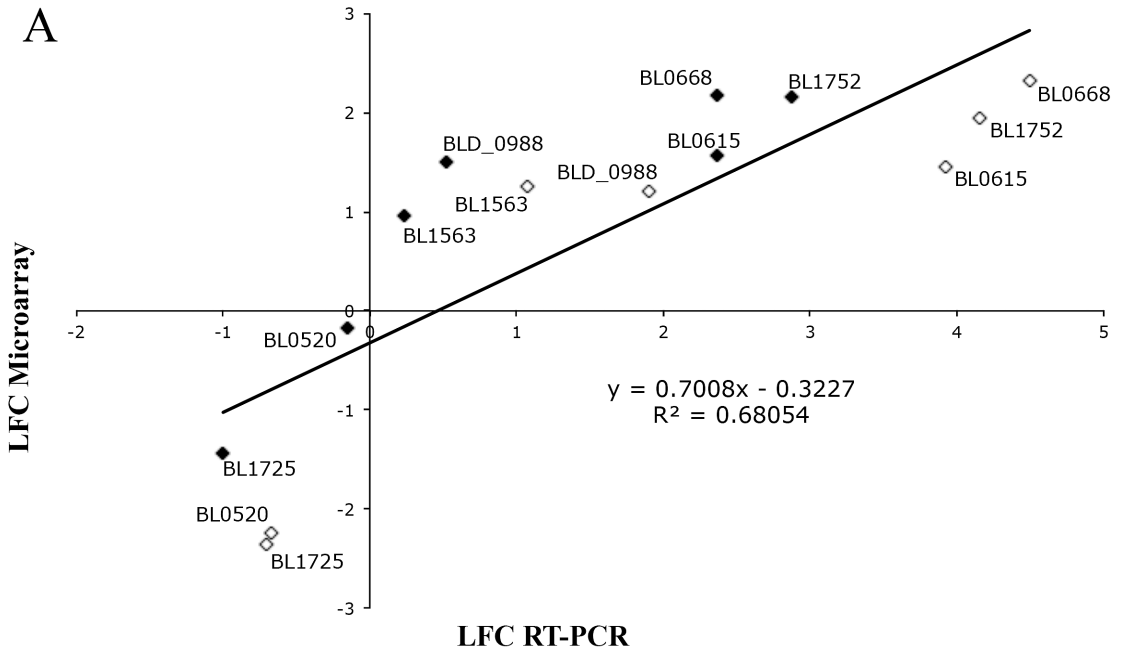
BL0646	Predicted S-adenosylmethionine-dependent methyltransferase involved in bacterial cell division	-0.70	NS	NS	NS
BL1011	Cell division transcription factor WhmD	NS	NS	1.27	1.66
BL1682	Cell division protein ftsH	NS	NS	NS	1.16
Poorly characterized, general function only					
BL0005	Predicted esponse regulator	1.91	1.14	NS	NS
BL0041	Predicted membrane protein	0.88	NS	NS	NS
BL0042	Predicted RNA-binding protein containing KH domain, possibly ribosomal protein	0.81	NS	NS	NS
BL0306	Predicted RNA-binding protein	0.73	NS	NS	NS
BL0690	Short-chain alcohol dehydrogenase of unknown specificity	1.31	1.12	0.95	NS
BL0721	Uncharacterized bacitracin resistance protein	0.82	NS	NS	NS
BL0743	putative membrane-associated GTPase	1.09	1.00	NS	NS
BL0765	Acetyltransferase	-1.47	NS	NS	NS
BL0782	Virulence protein	NS	0.78	NS	NS
BL0863	Hit Family Protein	NS	NS	0.96	0.95
BL0885	Predicted acyltransferases	-0.80	-1.08	NS	NS
BL0986	Response regulator with putative antiterminator output domain	1.04	NS	NS	NS
BL1000	Response regulators / Two-component response regulator	1.12	NS	NS	NS
BL1016	FKBP-type peptidyl-prolyl cis-trans isomerases 1	1.54	NS	NS	NS
BL1053	Hydrolase (HAD superfamily)	NS	NS	1.03	NS
BL1192	Metal-dependent hydrolase	NS	NS	NS	1.12
BL1181	Surface antigen	0.88	NS	NS	NS
BL1234	Abortive infection phage resistance protein	-0.79	-0.77	NS	NS
BL1278	Transporter	NS	1.12	NS	NS
BL1379	Predicted membrane GTPase involved in stress response	0.95	NS	NS	NS
BL1430	Multidrug resistance protein	NS	NS	NS	0.83
BL1575	NAD-dependent aldehyde dehydrogenases	1.07	NS	NS	NS
BL1627	Rrf2 family protein	1.05	NS	NS	NS

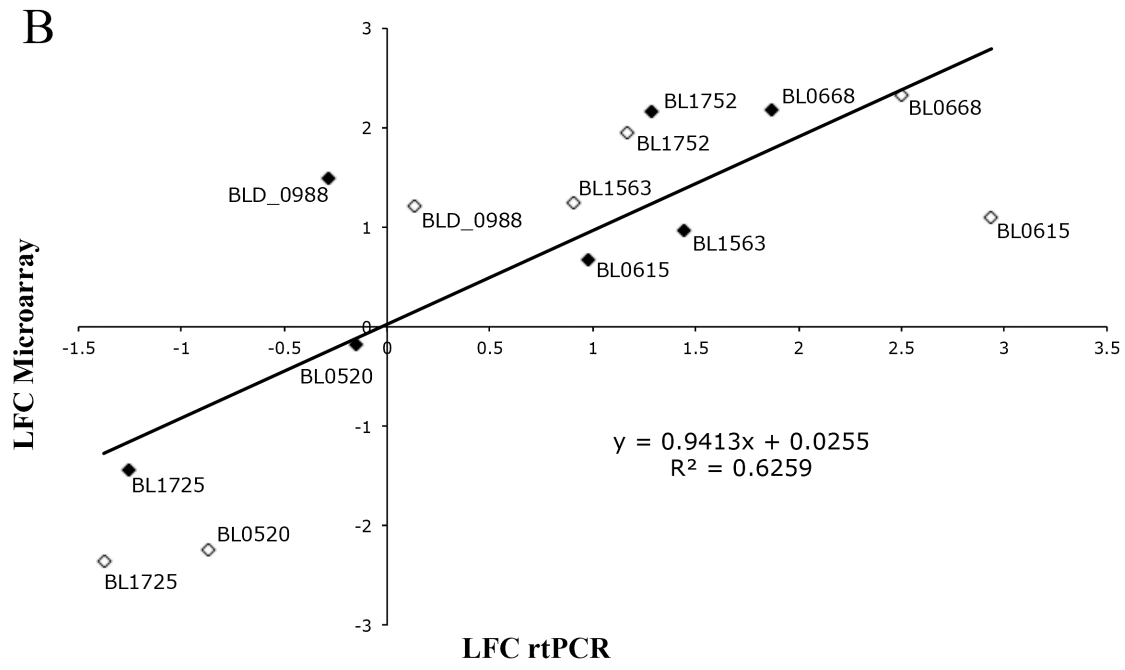
BL1725	Dioxygenase	-1.87	-2.03	NS	NS
BLD_0185	Hypothetical cytosolic protein/ putative addiction module killer protein	-1.42	-1.71	NS	NS
BLD_0254	Transporter	1.24	NS	NS	NS
BLD_0989	Membrane-bound transglycosylase	NS	NS	NS	1.16
BLD_0502	Carbon-nitrogen hydrolase family protein	0.85	NS	NS	NS
BLD_0970	ACT domain-containing protein	0.72	NS	NS	NS
BLD_1036	Predicted membrane protein	0.59	NS	NS	NS
Uncharacterized, hypothetical proteins					
BL0090	Hypothetical protein	0.86	NS	NS	0.78
BL0095	Hypothetical protein	0.76	0.85	NS	NS
BL0099	Hypothetical protein	NS	NS	NS	1.35
BL0120	Hypothetical protein	1.28	1.26	NS	NS
BL0121	Hypothetical protein	NS	NS	NS	1.41
BL0192	Hypothetical protein	-1.10	-1.03	NS	NS
BL0214	Hypothetical protein	-0.69	NS	NS	NS
BL0353	Hypothetical protein	-0.69	NS	NS	NS
BL0401	Hypothetical protein	1.58	1.10	0.89	0.88
BL0496	Hypothetical protein	-0.80	NS	NS	NS
BL0497	Hypothetical protein	-0.81	NS	NS	NS
BL0561	Hypothetical protein	-0.81	NS	NS	NS
BL0562	Hypothetical protein	NS	-1.24	NS	NS
BL0599	Hypothetical cytosolic protein	1.03	0.80	0.98	NS
BL0606	Hypothetical protein	-0.72	NS	NS	NS
BL0624	Hypothetical protein	-0.74	NS	NS	NS
BL0639	Hypothetical protein	-0.76	NS	NS	NS
BL0665	Hypothetical protein	-0.87	NS	NS	NS
BL0677	Hypothetical protein	-0.75	NS	NS	NS
BL0726	Hypothetical protein	1.08	NS	NS	0.89
BL0811	Hypothetical protein	-0.78	NS	NS	NS
BL0812	Hypothetical protein	-0.73	NS	NS	NS
BL0827	Hypothetical protein	1.38	NS	NS	NS
BL0913	Hypothetical protein	1.30	NS	NS	NS
BL0952	Hypothetical protein	1.75	1.48	NS	NS
BL1033	Hypothetical protein	0.71	NS	NS	NS
BL1084	Hypothetical protein	1.24	NS	NS	NS
BL1094	Hypothetical membrane protein	-1.98	-2.48	NS	-1.70
BL1134	Hypothetical protein	0.77	NS	NS	NS
BL1139	Hypothetical protein	1.10	0.92	NS	NS
BL1235	Hypothetical protein	-0.91	-0.79	NS	NS
BL1236	Hypothetical protein	-0.81	NS	NS	NS

BL1242	Hypothetical protein	-0.86	-0.86	NS	NS
BL1243	Hypothetical protein	-1.25	-1.33	NS	NS
BL1407	Hypothetical protein	0.86	NS	NS	NS
BL1557	Hypothetical protein	0.85	NS	NS	NS
BL1622	Hypothetical protein	-0.85	NS	NS	NS
BL1641	Hypothetical protein	-0.65	NS	NS	NS
BL1698	Hypothetical protein	-0.76	NS	NS	NS
BL1793	Hypothetical protein	0.96	NS	NS	NS
BL1806	Hypothetical protein	-0.64	NS	NS	NS
BL1813	Hypothetical protein	1.25	1.06	NS	NS
BLD_0575	Hypothetical protein	NS	NS	1.95	2.90
BLD_0108	Hypothetical protein	NS	NS	NS	1.04
BLD_0139	Hypothetical protein	NS	NS	NS	2.23
BLD_0224	Hypothetical protein	2.08	1.60	NS	1.03
BLD_0349	Hypothetical protein	-1.56	NS	NS	NS
BLD_0360	Hypothetical protein	0.88	NS	NS	NS
BLD_0516	Hypothetical protein	NS	1.13	NS	NS
BLD_0573	Hypothetical protein	-0.89	NS	NS	NS
BLD_0576	Hypothetical protein	NS	NS	NS	2.68
BLD_0617	Hypothetical protein	1.58	1.24	1.16	NS
BLD_0618	Hypothetical exported protein	1.52	1.46	1.21	1.04
BLD_0660	Hypothetical protein	0.81	NS	NS	NS
BLD_0661	Predicted membrane protein	1.05	NS	NS	NS
BLD_0662	Hypothetical protein	0.65	NS	NS	NS
BLD_0725	Hypothetical protein	-0.82	NS	NS	NS
BLD_0783	Hypothetical protein	1.05	NS	NS	NS
BLD_0788	Hypothetical protein	-0.88	NS	NS	NS
BLD_0897	Hypothetical protein	1.17	NS	NS	NS
BLD_0928	Hypothetical protein	-1.20	-2.40	NS	-1.88
BLD_0992	Hypothetical protein	1.11	NS	NS	NS
BLD_1262	Hypothetical protein	-0.78	NS	NS	NS
BLD_1370	Hypothetical protein	-0.83	NS	NS	NS
BLD_1415	Hypothetical protein	-0.86	NS	NS	NS
BLD_1419	Hypothetical protein	-0.80	NS	NS	NS
BLD_1521	Hypothetical protein	1.57	NS	1.40	NS
BLD_1509	Hypothetical protein	NS	NS	NS	-1.18
BLD_1736	Hypothetical protein	-0.88	NS	NS	NS
BLD_1748	Hypothetical protein	0.86	NS	NS	1.42
BLD_1749	Hypothetical protein	1.39	1.21	NS	NS
BLD_1761	Hypothetical protein	0.82	NS	NS	NS
BLD_1862	Hypothetical protein	-0.60	NS	NS	NS

[a]NS, Not Significant

FIG A-1 Correlation of fold change values from DNA microarray and real-time quantitative PCR results from *B. longum* NCC2705 (A) and D2957 (B). Fold change values were obtained for the 6 genes listed in Table 1. Symbols denote expression values from cells after 5 min (open diamonds) or 20 min (filled diamonds) exposure to 1.25 mM H_2O_2. The best-fit curve is shown along with the calculated equation and r^2 value


A
LFC Microarray
LFC RT-PCR
y = 0.7008x - 0.3227
R² = 0.68054
BL0668
BL1752
BL0668
BL1752
BLD_0988
BL0615
BLD_0988
BL0615
BL1563
BL1563
BL0520
BL1725
BL0520
BL1725


B
LFC Microarray
LFC rtPCR
y = 0.9413x + 0.0255
R² = 0.6259
BL1752
BL0668
BL0668
BL1752
BLD_0988
BL1563
BLD_0988
BL0615
BL1563
BL0615
BL0520
BL1725
BL0520
BL1725

APPENDIX B

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September 23, 2013

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Oberg TS, Ward RE, Steele JL, Broadbent JR. 2013. Genetic and physiological responses of *Bifidobacterium animalis* subsp. *lactis* to hydrogen peroxide stress. J. Bacteriol. 195:3743-3751

Oberg TS, Ward RE, Steele JL, Broadbent JR. 2012. Identification of plasmalogens in the cytoplasmic membrane of *Bifidobacterium animalis* subsp. *lactis*. Appl. Environ. Microbio. 78:880–884.

Oberg TS, Steele JL, Ingham SC, Smeianov VV, Briczinski EP, Abdalla A, Broadbent JR. 2011. Intrinsic and inducible resistance to hydrogen peroxide in Bifidobacterium species. J Ind Microbiol Biotechnol 38:1947–1953.

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Oberg TS, Steele JL, Ingham SC, Smeianov VV, Briczinski EP, Abdalla A, Broadbent JR. 2011. Intrinsic and inducible resistance to hydrogen peroxide in *Bifidobacterium* species. J Ind Microbiol Biotechnol 38:1947–1953.

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LABORATORY EXPERIENCE

Graduate Research Assistant: Utah State University 2007-present

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Research Assistant: EC Services Inc. 2003-2006

Laboratory Aid: Weber State University 2001-2006

EDUCATION

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Major: Microbiology.
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Ph.D. *November 2013* *Utah State University*
Major: Food Science with an emphasis on genetics and the physiological stress responses of bifidobacteria.

Advanced Training or Short Courses

Pathway Tools and BioCyc: Software and databases for pathway and genome data of metabolic engineering. Society of Industrial Microbiology workshop, July 2010.

Statistical Bioinformatics: Training in the statistical theory and analysis of microarry data, including training in the use of Bioconductor in the statistical platform R. Utah State University Department of Math and Statistics, Jan-May 2009.

Microbial Fermentation - Development & Scale-Up: Hands on instruction in microbial fermentation from bench scale to pilot scale including design and optimization of fermentation processes in batch and fed-batch cultures. Center for Integrated Biosystems, Utah State University, August 2008.

Gene Expression & Microarray Analysis: Advanced training in the main areas of microarray technology. The course focused on microbial as well as plant gene expression and emphasized theory, sample preparation, labeling, fluorescent imaging, microarray data analysis, and data mining. Hands on training in sample preparation and hybridization, including the Affymetrix platform. Center for Integrated Biosystems, Utah State University, May 2008.

AWARDS AND HONORS

2010 Outstanding Student Oral Presentation, Society for Industrial Microbiology Annual Meeting

2009 Outstanding Student Presentation, ASM Intermountain Branch Annual Meeting

2006 Outstanding Service Award Microbiology Department

2005 Paul and Carolyn Thompson Research Fellowship Award

2005 Lane Cedric Rolling Scholarship Recipient

2004 Neutraceutical Scholarship Recipient

2004 Denkers Family Undergraduate Research Fellow Award

RESEARCH EMPHASIS

Emphasis and interests: My current research areas center mainly on the genetics and physiology of probiotic bifidobacteria. This research utilizes DNA microarrays to study the transcriptional response of bifidobacteria to environmental stress, and analyses of the bacterial membrane fatty acids in response to environmental stress.

Publications in peer reviewed journals:

Oberg TS, Ward RE, Steele JL, Broadbent JR. 2013. Genetic and physiological responses of *Bifidobacterium animalis* subsp. *lactis* to hydrogen peroxide stress. J. Bacteriol. **195**:3743-3751

Oberg TS, Ward RE, Steele JL, Broadbent JR. 2012. Identification of plasmalogens in the cytoplasmic membrane of *Bifidobacterium animalis* subsp. *lactis*. Appl. Environ. Microbio. **78**:880–884.

Oberg TS, Steele JL, Ingham SC, Smeianov VV, Briczinski EP, Abdalla A, Broadbent JR. 2011. Intrinsic and inducible resistance to hydrogen peroxide in *Bifidobacterium* species. J. Ind. Microbiol. Biotechnol. **38**:1947–1953.

Oberg TS, Nakaoka K, Domek M, Oberg CJ. 2009. The Inhibition of nasal *Staphylococcus aureus* by lactic acid bacteria. J. Utah Acad. Sci. Arts Lett. 2009 77-85.

Barrangou R, Briczinski EP, Traeger LL, Loquasto JR, Richards M, Horvath P, Coûté-Monvoisin A-C, Leyer G, Rendulic S, Steele JL, Broadbent JR, Oberg T, Dudley EG, Schuster S, Romero DA, Roberts RF. 2009. Comparison of the complete genome sequences of *Bifidobacterium animali*s subsp. *lactis* DSM 10140 and Bl-04. J. Bacteriol. **191**:4144–4151.

Manuscripts Submitted and Under Review:

Oberg TS, Ward RE, Steele JL, Broadbent JR. Transcriptome analysis of *Bifidobacterium longum* strains that show a differential response to hydrogen peroxide stress. Applied and Environmental Microbiology.

Broadbent JR. Oberg TS, Hughes JE, Ward RE, Welker DE, Steele JL. Influence of polysorbate 80 and cyclopropane fatty acid synthase activity on lactic acid production by *Lactobacillus casei* ATCC 334 at low pH. Journal of Industrial Microbiology and Biotechnology.

Manuscripts in Preparation:

Broadbent JR, Brighton CJ, Oberg TS, Ward LJ, Young MJ, Ward RE, Steele JL. Influence of fat reduction on volatiles production and gene expression in *Lactococcus lactis* M70 in a model cheese serum.

Oberg CJ, Culumber MD, Oberg TS, Ortaki F, Broadbent JR, McMahon DJ. Genomic and physiological analysis of *Lactobacillus wasatchii* sp. *nov*., a late gas producing non-starter lactic acid bacteria isolated from aged cheddar cheese.

Oberg CJ, Culumber MD, Oberg TS, Ward RW, Broadbent JR, McMahon DJ, Steele JL. Using genomic and metabolic analysis to understand the dominance of *Lactobacillus curvatus* WSU1 as a non-starter lactic acid bacteria in aged cheddar cheese.

Grantsmanship:

Oberg*, T. S., M. Domek, Effect of Heat on Epithelial Cell Viability. WSU RS & PG Grant. Funded $3000 (May- August 2005)

Oberg, T. S., K. Nakaoka. Travel Grant for Presentation of Isolation and Characterization of Lactic Acid Bacteria That Inhibit *Staphylococcus aureus*. Paul and Carolyn Thompson Research Fellowship. Funded $1775 (July 2005)

Oberg, T. S., K. Nakaoka. Isolation and Characterization of Lactic Acid Bacteria That Inhibit *Staphylococcus aureus*. Denkers Family Undergraduate Research Fellow. Funded $3000 (May-August 2004)

Professional Presentations:

Oberg*, C.J., M.D. Culumber, T.S . Oberg, J. R. Broadbent, D. J. McMahon, and J. L. Steele. 2013. Comparative genome analysis of *Lactobacillus curvatus* strains isolated from cheese and fermented sausage. Poster presentation at the annual meeting of the American Dairy Science Association. July 8-12, Indianapolis, IN.

Oberg*, C.J., M.D. Culumber, T.S. Oberg, F. Ortakci, J. R. Broadbent, and D. J. McMahon. 2013. Genomic analysis of *Lactobacillus* WDC04, a novel species associated with late gas production in cheese. Poster presentation at the annual meeting of the American Dairy Science Association. July 8-12, Indianapolis, IN.

Oberg*, T. S., R. E. Ward, D. E. Welker, J. E. Hughes, J. L. Steele, J. R. Broadbent. 2012. Role of *Lactobacillus casei* cyclopropane fatty acid synthase (cfa) in lactic acid production at low pH, Poster presentation at the Society for Industrial Microbilogy Ann. Mtg., August 12-16, Washington D.C.

Oberg*, T. S., R. E. Ward, J. L. Steele, and J. R. Broadbent. Identification of ether-linked lipids in the cytoplasmic membrane of *Bifidobacterium animalis* ssp. *lactis.* 2011. Poster presentation at the 10th Symposium on Lactic Acid Bacterium, August 28-September 1, Egmond aan Zee, the Netherlands.

Oberg*, T. S., J. L. Steele, and J. R. Broadbent. Transcriptional response to hydrogen peroxide stress by *Bifidobacterium longum* strains BL-04 and DSM10140. 2011. Poster presentation at the 10th Symposium on Lactic Acid Bacterium, August 28-September 1, Egmond aan Zee, the Netherlands.

Oberg*, T. S., R. E. Ward, J. L. Steele, and J. R. Broadbent. Transcriptional and physiological response to hydrogen peroxide stress by *Bifidobacterium animalis* ssp. *Lactis*. 2011. Poster presentation at the 10th Symposium on Lactic Acid Bacterium, August 28-September 1, Egmond aan Zee, the Netherlands.

Oberg*, T. S., R. E. Ward, J. L. Steele, and J. R. Broadbent. Transcriptional and physiological responses of *Bifidobacterium animalis* ssp. *lactis* strains to hydrogen peroxide stress. 2011. Oral Presentation at the Ann. Mtg. American Dairy Science Association, July 10-14, New Orleans, Louisiana.

Oberg*, T. S., R. E. Ward, J. L. Steele, S. Ingham, J. R. Broadbent. Differential Transcriptional Response and physiological differences of *Bifidobacterium animalis* ssp. *lactis* Strains to Hydrogen Peroxide Stress, 2010. Oral and Poster Presentation at 6th International Symposium on Probiotics and Health, October 28-29, Montreal, Canada.

Oberg*, T. S., R. E. Ward, J. L. Steele, S. Ingham, J. R. Broadbent. Differential Response of *Bifidobacterium animalis* ssp. *lactis* Strains to Hydrogen Peroxide Stress. 2010. Oral and Poster Presentation at the Society for Industrial Microbilogy Ann. Mtg., August 1-5, San Francisco, California.

Oberg*, T. S., S. C. Ingham, J. L. Steele, and J. R. Broadbent. Transcriptional Stress Responses to Hydrogen Peroxide in *Bifidobacterium longum*. 2010. Oral Presentation at the Ann. Mtg. American Dairy Science Association, July 11-15, Denver, Colorado.

Oberg*, T. S., R. E. Ward, S. C. Ingham, J. L. Steele, and J. R. Broadbent. Physiological Stress Responses to Hydrogen Peroxide in *Bifidobacterium animalis* ssp. *lactis*. 2010. Oral presentation at the ASM Intermountain Branch Annual Meeting. April 10, Brigham Young University, Provo, Utah.

Oberg*, T. S., S. C. Ingham, J. L. Steele, and J. R. Broadbent. Intrinsic resistance and stress responses to hydrogen peroxide in bifidobacteria. 2009. Oral presentation at the Ann. Mtg. American Dairy Science Association, July 12-16, Montreal, Canada.

Oberg*, T. S., M. Domek, E. Call. Effect of Heat on Epithelial Cell Viability. 2007. Oral presentation at the Ann. Mtg. Utah Academy of Science, Arts and Letters, April 7, Snow College, Ephraim, Utah.

Oberg*, T. S., M. Domek, E. Call. Effect of Heat on Epithelial Cell Viability. 2006. Oral presentation at the ASM Intermountain Branch Annual Meeting. March 18, Brigham Young University, Provo, Utah.

Oberg*, T. S., K. Nakaoka, C. Oberg. The Inhibition of Nasal *Staphylococcus aureus* by Lactic Acid Bacteria. 2005. Poster Presentation at the International Union of Microbiological Societies. July 23-28, San Francisco, California.

Oberg*, T. S., E. Call, B. Oberg. Wheelchair Aging Studies Utilizing Fecal Coliforms. 2005. Oral Presentation at the Ann. Mtg. Utah Academy of Science, Arts and Letters, April 15, Utah Valley State College, Orem, Utah.

Oberg*, T. S., K. Nakaoka, C. Oberg. The Inhibition of *Staphylococcus aureus* by Lactic Acid Bacteria. 2005. Oral presentation at the ASM Intermountain Branch Annual Meeting. March 12, Weber State University, Ogden, Utah.

Oberg*, T. S., C. Oberg, K. Oberg. Phage Suppression of Bioterrorism Agents: A Model System. 2004. Poster presentation at the ASM Intermountain Branch Annual Meeting, March 27, Eastern Idaho Technical College, Idaho Falls, Idaho.